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Demonstration of Eaton's Agent in Tissue Culture.* (26732)

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Many problems of studying the Eaton agent of primary atypical pneumonia have related to its limited host range and the lack of convenient methods to indicate its presence in a test system. The indirect fluorescent antibody procedure was adapted to localization of the agent in the chick embryo by Liu(1). More recently Chanock, *et al.* (2) have demonstrated that propagation in tissue culture systems is possible; they were unable to visualize the agent directly, however, and it was necessary to subculture the tissue culture materials in chick embryos with application of Liu's methods to identify the agent. This report concerns successful direct localization of Eaton's agent in tissue culture. The data to follow amplify the observations of Marmion and Goodburn(3) which have suggested a relationship between the agent and pleuropneumonia-like organisms.

Materials and methods. The Mac strain of Eaton's agent, obtained from Dr. Chien Liu and passaged 8 times in chick embryos in this laboratory, was used in these studies. Rhesus monkey kidney cell flask cultures were obtained from Microbiological Associates, removed from the glass with 0.25% trypsin, and resuspended in growth medium (Hanks' BSS 90%, inactivated calf serum 10%, lactalbumin hydrolysate 0.5%, penicillin 1,000 units/ml). Secondary monolayers of these cells were prepared on 6 × 22 mm

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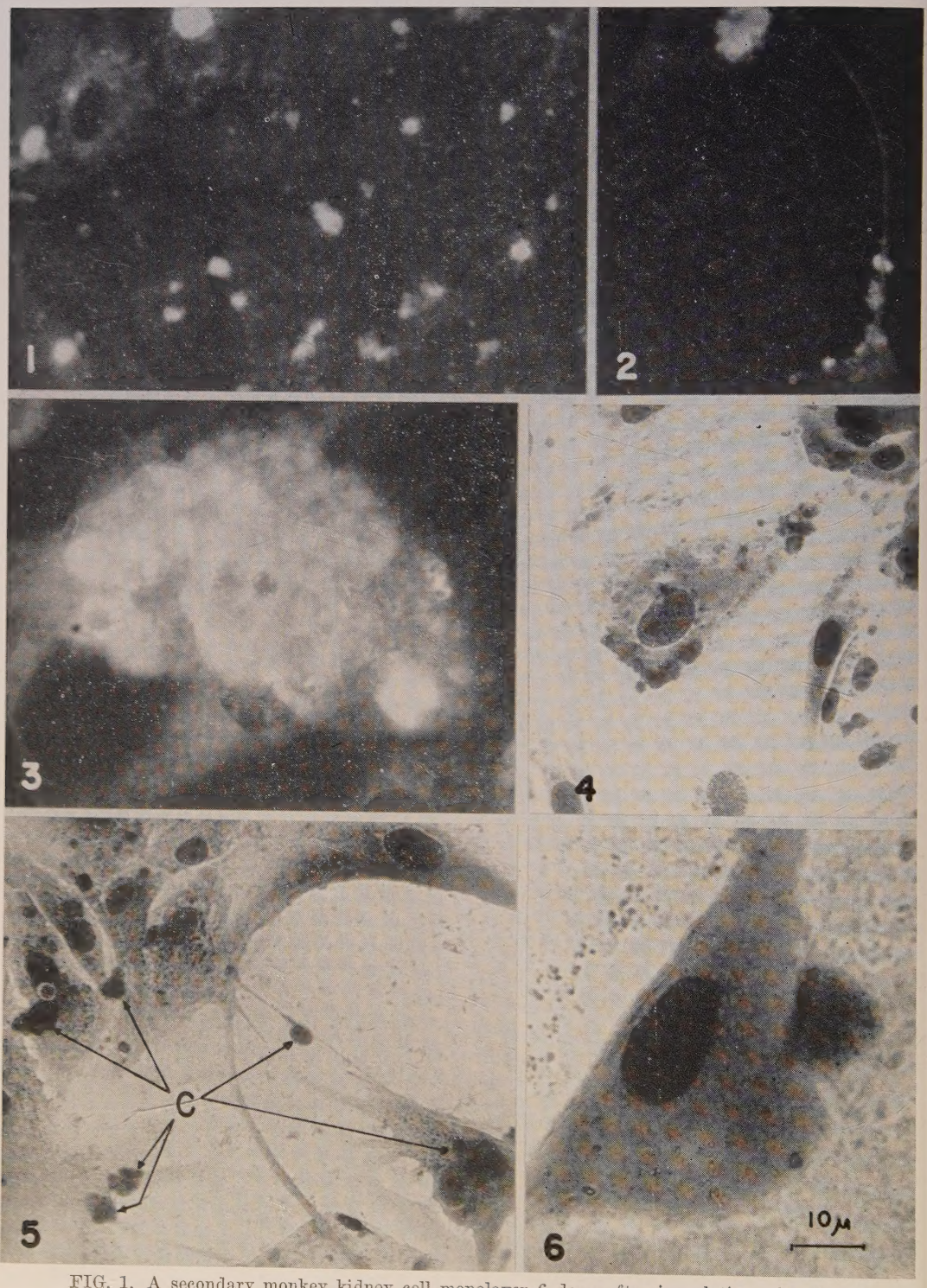

FIG. 1. A secondary monkey kidney cell monolayer 6 days after inoculation with Eaton's agent. Rounded, granular growths are distributed at random over surface of culture. Fluorescent antibody technic. ×430.

coverslips in screw cap tubes, and before inoculation the fluid was changed to a special maintenance medium (Eagle's medium with double amino acids and vitamins 95%, inactivated chicken serum 5%, l-glutamine 0.025%, penicillin 1,000 units/ml, and sufficient $NaHCO_3$ solution to approximate pH 7.4). Infected chick embryo lung suspension was centrifuged 1500 rpm for 10 minutes to clarify, dilutions were prepared in maintenance medium at 4°C, and tubes were inoculated to contain a 10^{-2} final dilution of the stock agent. After various periods of incubation in stationary racks at 32°C the coverslips were harvested, air-dried at room temperature, fixed in acetone for 10 minutes, and stained with either the indirect fluorescent antibody technic as previously described(4) or an intensified Giemsa method(3). Antisera were obtained from a serum collection of primary atypical pneumonia cases occurring in the Cleveland area in 1947-48, or by immunization of rabbits with stock agent as described by Liu(1). Previous titrations using infected chick embryo lung sections (4) revealed that the human convalescent sera contained antibody at dilutions of 1:320-1:640, while the rabbit antiserum had a titer of 5120.

Results. Observations of unfixed cultures. Under the conditions imposed the onset and character of cytopathic effects were variable. The changes consisted most often of the development of plaques of yellowish, granular, refractile cells with ultimate retraction and detachment from the glass. In some experiments these changes could be seen as early as 4 days, while in others they were not apparent until 10 days following inoculation of the cell sheets; at times no specific changes could be seen as late as 14 days, by which time the picture was often confused by the emergence of simian agents. It became apparent early in the course of these investigations that this method of detecting the presence of the Eaton agent was unreliable, and attention was turned to the use of the indirect fluorescent antibody technic.

Observations by means of fluorescent antibody staining. Coverslip cultures were harvested at various times following inoculation, and stained with sera known to contain fluorescent-stainable antibody against Eaton's agent. Antiserum dilutions of 1:10 were prepared in phosphate buffered saline (pH 7.2) and inactivated by heating at 56°C for 30 minutes. Controls for the reaction consisted of the following: 1) infected and uninoculated cell cultures stained with known-negative antisera (human acute or normal rabbit specimens); 2) cultures inoculated with normal chick embryo lung suspension stained with human convalescent serum or rabbit antiserum; and 3) normal and infected cultures stained only with the fluorescein-conjugated goat antirabbit or antihuman globulin solutions.

The changes to be described were seen in infected cultures stained either with human convalescent sera or rabbit antiserum, but not in any of the control systems. Beginning at 2 or 3 days after inoculation, and increasing until destruction or degeneration of the cultures at 14 days, brightly stained, rounded, granular structures were evident. The appearance of an intact culture 6 days after inoculation is illustrated in Fig. 1. Fig. 2 shows an infected monolayer after 10 days' incubation, at which time partial destruction of the cell sheet had occurred. The plane of focus of these structures under high magnification revealed them to be at the surface

FIG. 2. A partially destroyed cell sheet, 10 days after inoculation, with brightly stained structures on edges of cells and upon cytoplasmic processes. ×430.

FIG. 3. A large mass of antigenic material occurring 10 days after inoculation, which is composed of a cluster of smaller rounded units. ×430.

FIG. 4. Companion culture to that in Fig. 1, stained by the Giemsa method. Pink, granular structures are scattered across the surface of the large cell in the center of the field with peripheral aggregation. ×200.

FIG. 5. Companion culture to that in Fig. 2 showing partial destruction and retraction of cell sheet. Clusters of the rounded structures seen earlier are present (C), and all cells in the field now show evidence of infection. Giemsa stain. ×200.

FIG. 6. A single rounded cell bearing a colony on cell wall. The finely particulate nature of the growth is just discernible. Giemsa stain. ×900.

of cells, in intercellular spaces, on the edges of cells at the periphery of the monolayers, and upon long cytoplasmic processes of cells (Fig. 2). They were always in direct association with cells, however. These structures seemed fixed to the cell sheet since they did not wash off during the multiple agitated rinses of the staining procedure. Late in the course of infection much larger aggregates of fluorescing material were present after staining (Fig. 3), but these were composed of clusters of the smaller, rounded units. Cells were frequently seen which contained minute specifically-stained particles; it was impossible to determine if these were intracytoplasmic or on the surface of the cells. Because of the difficulties of visualizing fine detail with the fluorescence microscope, study of companion cultures stained by the Giemsa method was undertaken for further evaluation.

Observations by means of Giemsa staining. Changes comparable to those seen with the fluorescence microscope could be found by examination of infected cultures stained with an intensified Giemsa procedure (Fig. 4 and 5). These changes were not seen in either normal secondary monkey kidney cell cultures or in cultures inoculated with normal chick embryo lung suspension. Study of the alterations with the oil immersion objective revealed that the rounded structures associated with cells were composed of forms near the limit of resolution which stained light pink, and appeared to be predominantly rounded in shape (Fig. 6). Clusters of the minute forms averaged 10 μ in diameter as measured on photographic enlargements with appropriate corrections for degree of magnification, or more directly by use of a calibrated ocular micrometer.

Discussion. It has recently been shown that growth of the Eaton agent is inhibited by gold salts, and that minute cocco-bacilli are present in infected chick embryos in areas corresponding to those containing the fluorescent-stainable antigen(3). This information coupled with the size of the agent (5), range of antibiotic sensitivity(3), and the agent's stability characteristics(6) all are quite similar to those of the family of pleuropneumonia-like organisms (PPLO). The data presented revealed the development of extracellular growths in colonial configuration after inoculation of secondary monkey kidney cell cultures with Eaton's agent. The serological reaction of these colonies was indistinguishable from that of the same stock agent identified in the chick embryo. Carski and Shepard(7) have demonstrated PPLO in tissue culture systems using the fluorescent antibody technic, and their descriptions are strikingly similar to the findings with Eaton's agent reported above. This accumulated evidence strongly suggests that the Eaton agent is a member of, or shares many properties of, the genus *Mycoplasma.*

The methods described make possible experimentation with the agent directly in tissue culture without the need for an additional indicator system. Work is now in progress to evaluate application of these technics to further study of the growth and other biologic characteristics of the agent.

Summary. The Eaton agent was localized in secondary monkey kidney cell cultures by means of fluorescent antibody staining employing either human atypical pneumonia convalescent sera or rabbit antiserum. Morphologic study of material stained by the Giemsa method revealed the occurrence of extracellular growths resembling colonies with an average diameter of 10 micra. These findings plus other characteristics of the agent summarized suggest an intimate relationship to organisms of the genus *Mycoplasma.*

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Autoradiographic Studies of Cell Proliferation in the Periosteum of Intact and Fractured Femora of Mice Utilizing DNA Labeling with H^3-Thymidine.*† (26733)

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Opinions on the origin of periosteal cells differ(1). The periosteum consists of 2 layers, an outer layer of fibroblasts and an inner layer of pre-osteoblasts and osteoblasts. The origin of the preceding cell types is of fundamental interest. Apparent morphologic transitions between various cell types exist (Fig. 1). Therefore, it is difficult to tell which cells produce others without a marker that is permanent and is carried through the transitions. Such a marker is tritiated thymidine which labels DNA during replication prior to mitosis and is visible by autoradiographic methods for 5 to 6 cell generative cycles(2, 3,4). The availability of such a marker should make it possible to determine the sequential relations between pre-osteoblasts, osteoblasts, osteoclasts, osteocytes, the chondrocytic series, and fibroblasts.

The aim of these experiments is: 1. To determine the original cell types participating in fracture repair, and 2. To find out whether a common progenitor of these cells exists.

The normal cellular complement of the skeletal system has been recently studied autoradiographically with tritiated thymidine during growth and aging(5).

Methods and materials. A total of 45 female mice of the Brookhaven National Laboratory strain of Swiss Albino were divided into 5 age groups, namely 1, 5, 8, 26 and 52 weeks of age. The right femur was fractured by digital pressure at the mid-diaphyseal aspect, under ether anaesthesia. The left leg was used as a control. It has been established from normal and fracture studies previously reported(5,6) that no qualitative or quantitative changes are observed in tritiated thymidine autoradiographs of non-fractured femora when the opposite limbs have been fractured. Animals were killed 24 hours, 1 week and 2 weeks post-fracturing. One hour prior to killing, the mice received a subcutaneous injection of 0.5 μc of tritiated thymidine per gram of body weight. Tissues were fixed in acetic-alcohol for 3 hours, followed by an additional 24 hours of fixation in formol-saline and washed for 24 hours with running tap water. Decalcification was carried out in a 10% solution of Versene. Paraffin sections were cut at 5 μ and subsequently covered with a liquid emulsion of Kodak NTB_3 and exposed for 20 days in a cold, dry atmosphere. Preparations were stained with Harris hematoxylin after developing. Additional histological sections were stained with hematoxylin and eosin.

Measurements of the thickness of the periosteum at each age were made with an ocular micrometer.

Results and discussion. "Flash labeling" by injecting H^3TDR and then killing the animals before sufficient time has elapsed to permit labeled cells to divide, gives one an estimate of the proliferative rate at that instance by marking the fraction of cells that were synthesizing DNA preparatory to the next mitosis.

In the periosteum of normal intact mice, pre-osteoblasts are "flash" labeled more frequently than osteoblasts or fibroblasts. Examples are seen in Fig. 2. Osteocytes do not "flash" label. With increasing age the fraction of cells "flash" labeled diminishes. The fractions labeled in non-fractured femora were relatively constant 1 day, 1 and 2 weeks after fracture of the opposite femur.

Following fracture of the femur the fraction of cells labeled in the periosteum 24 hours after fracture was greater throughout the entire shaft (Fig. 3). One week after fracture, labeling was most prevalent at the fracture site but was less than after 24 hours.

* Research supported by U. S. Atomic Energy Commission.

† Work was presented in part at Seventh International Congress of Anatomists, New York City, April 11, 1960.

Two weeks after fracture the periosteal labeling was similar to the control leg except at the fracture site. After fractures there is more abundant labeling of pre-osteoblasts than osteoblasts and only a slight increase in labeling of fibroblasts.

Striking changes are observed in the periosteum with increasing age. The total thickness decreases from about 136 μ to 48 μ and the osteogenic layer from 77 to 14 μ with increasing age (Table I). The per cent reduction in thickness is 42% in the fibrous layer and 81% in the osteogenic layer between 1 and 52 weeks of age. The diminution in thickness is due in part to the presence of fewer and smaller cells. However, following trauma, the periosteum can respond at all ages by increased cell production with resultant thicker periosteum. The decreased rate of callus formation in fractures of older animals corresponds to the increased time needed to produce sufficient cells to repair the fracture.

These autoradiographic studies may be considered as "flash labeled" since the one hour interval between injection of the DNA label and killing of the animals does not permit cell division to occur. Thus the proliferative potentials of all cells at that instance are recorded. Pre-osteobasts, osteoblasts and fibroblasts possess proliferative potentials of decreasing order. Osteocytes and osteoclasts are presumably non-proliferative "sterile cells" produced only by transformation or fusion of precursor cells(7).

From these studies it is highly unlikely that fibroblasts produce osteogenic cells or that osteogenic cells produce fibroblasts. It appears that fibroblasts either have an autonomous origin in part or are in part produced by migration of mononuclear cells from the blood(8).

Tentatively it is believed that pre-osteoblasts produce chondrogenic cells and osteoblasts in fracture repair. The fibrous layer of the periosteum apparently contributes little if any to the osteogenic layer, whereas, during fracture repair the thickening osteogenic layer is produced by production of new osteoblasts (Fig. 1, No. 3).

Actual cellular flow rates and pathways

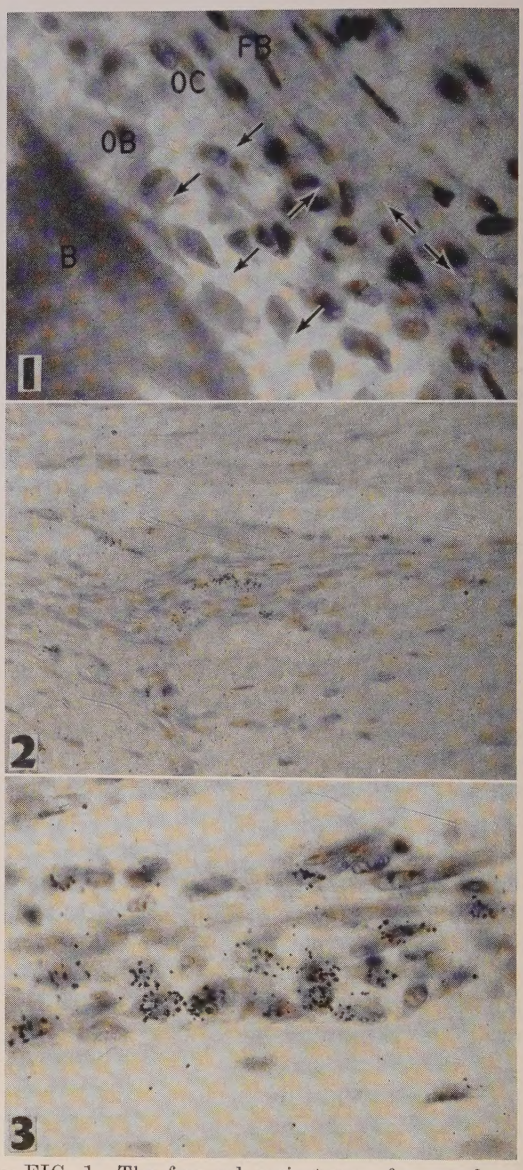

FIG. 1. The femoral periosteum of a new-born mouse is shown depicting the possible origin and pathways of various cell types. (FB) represents the fibrous layer of periosteum, (OC) preosteoblasts, (OB) osteoblasts, and (B) cortical bone. (1) shows formation of pre-osteoblasts from fibroblasts and osteoblasts from pre-osteoblasts. (2) indicates formation of osteoblasts and fibroblasts from pre-osteoblasts. (3) shows that each cell type can contribute to each compartment and that the pre-osteoblasts serve as the progenitor pool for the supply of osteoblasts not fibroblasts.

FIG. 2. Autoradiograph of femoral periosteum of a 1-wk-old mouse labeled with tritiated thymidine. Hematoxylin stained. ×100.

FIG. 3. Autoradiograph of femoral periosteum of a 1-wk-old mouse, 32 hr after fracture, heavily labeled with tritiated thymidine. Hematoxylin stained. ×320 oil immersion.

TABLE I. Average Thickness of Mouse Periosteum (in $\mu \pm$ AD) Taken from the Anterior Aspect of the Mid-Shaft of the Femur.*

Age (wk)	1	5	8	26	52
Osteogenic layer	76.9 ± 17.5	53.9 ± 11.3	32.0 ± 6.3	18.5 ± 7.7	14.1 ± 10.5
Periosteum (fibrous + osteogenic layers)	136.3 ± 20.9	90.3 ± 15.3	87.6 ± 14.3	60.8 ± 10.2	48.1 ± 13.2

* 15 areas were measured in each case.

can only be proved by serial killing after a single injection of label.

Summary. An autoradiographic study of cell proliferation in intact and fractured femoral periosteum of mice using H^3-thymidine showed that osteogenic cells are a relatively quiescent cell population awaiting a signal for proliferation and transformation as in fracture repair. Osteogenic cells constitute a self-sustaining cell population, which becomes diminished in size with increasing age. Osteoblasts are in part self-reproducing and in part are produced by transformation of pre-osteoblasts.

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Epinephrine Metabolism in Phenylketonuria.* (26734)

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A possible disturbance in the metabolism of epinephrine in phenylketonuria has been suggested. Cawte(1,2) has demonstrated an increased rise of blood pressure following an intravenous dose of adrenalin among phenylketonurics and attributed this to an impairment of epinephrine production. Weil-Malherbe(3) has shown a decrease of plasma epinephrine and norepinephrine in various forms of mental defect, with phenylketonurics being among the lowest.

The present paper will describe the decrease of dopamine, norepinephrine, and epinephrine in phenylketonuria and discuss evidence for the *in vivo* inhibition of dopa decarboxylase by phenylalanine derivatives.

Materials and methods. Two groups of phenylketonurics were studied. Eight patients† (ages 9-15) had received no specific treatment and 6 patients (ages 3-7) had been treated with low-phenylalanine diet from 6 months to 4 years. Identical studies were carried out in 8 normal and 8 mentally-retarded controls of comparable age and sex to the untreated phenylketonurics.

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† The kind cooperation of Dr. Tillman and staff members of Dixon State School is gratefully acknowledged.

TABLE I. Summary of Values in Controls and Phenylketonurics.

	Controls		Phenylketonurics		
	Normal (8)	Retarded (8)	Untreated (8)	Treated (6)	P*
Plasma					
Phenylalanine (mg %)	1.92 ± 1.83	2.02 ± .76	37.00 ± 4.40	8.46 ± 4.74	<.001
Tyrosine (")	1.95 ± .34	2.08 ± .48	1.93 ± .42	1.05 ± .19	.8
Norepinephrine (μg/l)	3.00 ± .75	3.13 ± .97	1.30 ± .46	1.93 ± .57	.01–.02
Epinephrine (")	1.81 ± .72	1.91 ± .56	.78 ± .23	1.27 ± .40	.02–.05
Urine					
Dopamine (μg/24 hr)	52.97 ± 4.22	56.35 ± 5.13	26.65 ± 3.18	38.61 ± 2.77	<.001
Norepinephrine (")	9.62 ± 1.70	10.49 ± 3.30	3.46 ± 1.14	6.63 ± 1.02	.005–.01
Epinephrine (")	5.94 ± .39	6.42 ± 1.88	1.70 ± .67	3.33 ± .90	<.001

Numbers in parentheses indicate number of subjects tested. Values expressed as means and stand. dev.

* t test of difference between controls and untreated phenylketonurics.

Twenty-four hour urine specimens were collected in dark bottles containing 10 ml 6N HCl. Urine norepinephrine and epinephrine were determined by the method of von Euler and Lishajko(4) and dopamine by the method of Carlsson and Waldeck(5). During the collection, a blood sample was obtained using heparinized syringes. Plasma phenylalanine was determined by the method of LaDu and Micheal(6), tyrosine by the method of Udenfriend and Cooper(7), and norepinephrine and epinephrine by the method of Price and Price(8).

Results. The data are summarized in Table I. No significant difference exists between the controls of normal intelligence and those who are mentally retarded and the 2 groups may be treated as one. While plasma phenylalanine levels were markedly elevated in the untreated phenylketonurics, no alterations in plasma tyrosine levels could be detected. The decrease of plasma tyrosine in the treated phenylketonurics could in part be attributed to a relative deficiency of tyrosine in the low-phenylalanine diet(9).

There is a uniform decrease of epinephrine-like compounds in the untreated phenylketonurics. In the plasma, the decrease of norepinephrine ($.02<P<.01$) and epinephrine ($.05<P<.02$) is moderately significant. In the urine, the decrease of dopamine ($P<.001$), norepinephrine ($P<.005$) and epinephrine ($P<.001$) is highly significant.

In all instances, this decrease is reversible when the phenylketonuric is treated with a low-phenylalanine diet. The inverse relationship between individual values for plasma phenylalanine and epinephrine-like compounds is shown in Table II. The negative correlation coefficient in each group is highly significant ($P<.001$).

Discussion. Holtz(10) has shown that the primary pathway of epinephrine formation is as follows:

$$\text{Tyrosine} \longrightarrow \text{Dopa} \xrightarrow[\text{Decarboxylase}]{\text{Dopa}} \text{Dopamine} \longrightarrow \text{Norepinephrine} \longrightarrow \text{Epinephrine}$$

A decrease of dopamine, norepinephrine, and epinephrine in phenylketonuria could be caused either by a deficiency of tyrosine(1) or a decrease of dopa decarboxylase(11). In the present study, levels of tyrosine in the plasma appear to be unaltered by the excessive phenylalanine. Although the decrease of epinephrine-like compounds could be caused by a failure of tyrosine to dopa, or by the use of an alternative pathway, it is most likely due to a decrease of dopa decarboxylase. Previously, Hartman *et al.*(12) and

TABLE II. Correlation between Plasma Phenylalanine and Various Epinephrine-Like Compounds.

Compound	Correlation coefficient*
Plasma — Norepinephrine	–.82
Epinephrine	–.65
Urine — Dopamine	–.73
Norepinephrine	–.73
Epinephrine	–.71

* $P < .001$ in all instances.

Fellman(13) have demonstrated *in vitro* inhibition of dopa decarboxylase by phenylpyruvic, phenyllactic, and phenylacetic acids. Since these derivatives of phenylalanine are known to be present in excessive quantities in phenylketonuria and this change is reversed by a diet low in phenylalanine content (13), the present data would appear to provide evidence for the *in vivo* inhibition of dopa decarboxylase.

Pare *et al.*(14) have indicated that the decrease of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in phenylketonuria is due to an inhibition of 5-hydroxytryptophan decarboxylase by phenylalanine metabolites. These findings have recently been confirmed in normal rats made phenylketonuric by feeding excessive amounts of both phenylalanine and tyrosine(15). Studies carried out *in vitro* by Yulwiler(16) and Hess *et al.* (17) have indicated that the pyridoxal-phosphate dependent decarboxylases may represent closely related or identical enzyme systems. The similarity between the changes caused by the derivatives of phenylalanine upon 5-hydroxytryptophan and dopa decarboxylase would seem to provide further *in vivo* evidence for this hypothesis.

Summary. Data have been presented showing a decrease of norepinephrine and epinephrine in the plasma and of dopamine, norepinephrine, and epinephrine in the urine of phenylketonuric children. These changes were reversed when the patients were treated with a low-phenylalanine diet. Since plasma tyrosine levels remained unchanged, this decrease appears to be caused by inhibition of dopa decarboxylase by the derivatives of phenylalanine. This would provide further *in vivo* evidence that the pyridoxal-phosphate dependent decarboxylases represent related or identical enzyme systems.

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Effects of Vitamin B_{12} and Methionine on Excretion of Formiminoglutamic Acid by the Chick.* (26735)

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Young rats fed a diet deficient in Vit. B_{12} were found by Silverman and Pitney(1) to excrete large amounts of formiminoglutamic acid (FGA), a metabolite of histidine that normally is excreted in negligible amounts in urine. They found that supplements of either Vit. B_{12} or methionine caused a drop in FGA

* Part of these data were presented at Fifth International Congress on Nutrition, Sept. 1-7, 1960, Washington, D. C.

TABLE I. Effect of Vit. B_{12} Deficiency upon Excretion of Formiminoglutamic Acid.*

Vit. B_{12}, mg/kg diet	Body wt ± S.E., g	Histidine intake ± S.E., mmole/day	μmoles FGA ± S.E.	
			Per day	Per mmole histidine
0	114 ± 3.3	1.1 ± .07	113 ± 13.9	108 ± 14.5
.1	215 ± 8.8	2.0 ± .11	20 ± 5.0	11 ± 2.7

* Avg of values from 3 exp., total of 21 chicks for each treatment, 22 days of age.

excretion to low or zero levels. This Vit. B_{12}-methionine relationship in the rat was closely analogous to that observed by Fox *et al.*(2,3) in young chicks; *i.e.*, growth was poor in the absence of Vit. B_{12} but good upon supplementation of the diet with either the vitamin or methionine. The diets used in both the rat and the chick studies contained high levels of fat and were borderline in the sulfur-containing amino acids. FGA excretion pattern of the Vit. B_{12} deficient chick was investigated because it seemed that this parameter of deficiency could be useful in elucidating some of the interrelationships between Vit. B_{12}, methionine, and fat. It was found that the Vit. B_{12} deficient chicks excrete large amounts of FGA and that this excretion rate is markedly reduced by methionine.

Methods. Day-old female New Hampshire chicks were placed in electrically heated batteries with screen wire floors. They were fed diet C62(4), a purified soybean protein diet that contained 24% of fat. Vit. B_{12} was omitted from the diet except as indicated in Table I. After 3-5 wks, the chicks were placed in individual cages and the excreta were collected and assayed for FGA as previously described(4,5). The number of chicks per experimental group varied between 5 and 8. During the collection periods, L-histidine HCl was added to the diet at a level of 1% to accentuate the differences in FGA excretion. Supplements of methionine and related compounds were incorporated into the diet for 24 hr intrevals as indicated in Tables II and III. In the experiments reported in Table IV, chicks were raised on a Vit. B_{12} deficient diet exactly like C62 except that fat content was lowered to 4%; glucose was added to compensate for the weight of fat (200 g/kg of diet) that was omitted. FGA excretion was determined while the chicks received the low level of dietary fat and also during the 2 days following an increase in dietary fat to 24%. In some experiments food intake was restricted on certain days. When this was done, the chicks received one-third of the day's ration at the beginning of the day and the remaining two-thirds 8 hr later.

In addition to colorimetric assays for FGA many extracts of excreta were also subjected to paper chromatography and paper electrophoresis. Components of the extract were separated in duplicate on Whatman 3MM paper by ascending chromatography for 16 hr. The developing solvent was the butanol phase which separated after mixing n-butanol 40%, glacial acetic acid 10%, and water 50% by volume. The strips were dried and one strip for each sample was exposed to the atmosphere over ammonium hydroxide for 1 hr to hydrolyze the formimino group from

TABLE II. Effect of Methionine Supplementation for One Day upon Excretion of FGA by Vit. B_{12} Deficient Chicks.*

Day	DL-methionine, % of diet	Body wt, g	Histidine intake, mmole/day	μmoles FGA	
				Per day	Per mmole histidine
1	0	203	2.0	103	52
2	0	212	1.9	111	58
3	1	231	2.6	14	5
4	0	232	1.8	270	150
5	0	239	1.9	121	64

* Mean values for 15 chicks from 2 experiments. Chicks were 31 days of age on day 1.

TABLE III. Effect of Compounds Related to Methionine upon Excretion of FGA by Vit. B_{12} Deficient Chicks.* (μmoles FGA/day/mmole histidine intake.)

Day	Supplement†	DL-methionine‡	DL-homocysteine thiolactone HCl	DL-homocystine	L-cystine	Choline chloride	Betaine
1	0	67	113	39	35	68	46
2	0	72	111	34	51	45	59
3	+	35	111	39	50	48	72
4	0	211	113	68	66	74	60
5	0	120	103	46	43	44	48
No. of chicks		5	7	13	5	13	12

* When more than 7 chicks received a given supplement, the results are the avg of 2 experiments; ages ranged between 21 and 31 days.

† The methionine supplement (Table II) was incorporated at a level of 1% of the diet; all supplements in this table were equimolar or equivalent in methyls (choline and betaine) to methionine.

‡ Food intake of each chick was limited on day of methionine supplementation to amount of diet consumed the previous day.

the FGA. Excess ammonia was dissipated from the strips by forced air at room temperature for ½ hr. All strips were then dipped in a solution of 0.2% ninhydrin in acetone. FGA does not stain with ninhydrin whereas the glutamic acid formed during hydrolysis stains blue. Other pairs of strips were sprayed with the same alkaline ferricyanide-nitroprusside reagent and sodium borate solution as used for the colorimetric assay(5). These reagents form a pink color with compounds having a formimino group. Electrophoresis was carried out with the pyridine-acetate buffer of Knowles(6) in Spinco Model R migration chambers at 400 volts for two and one-half hr. A sample stripper was used to apply 0.01 ml of sample across each strip of 3 cm wide Whatman 3MM paper. The same volume of a standard solution containing 1 μmole FGA/ml was used as a control. Following electrophoresis, the strips were dried and cut in half longitudinally. One of each pair was exposed to ammonia vapor and then both strips were stained in the same manner as described above.

Results. The Vit. B_{12} deficient chicks grew slowly and excreted more than 5 times as much FGA as chicks in the control group receiving Vit. B_{12} (Table I). This difference was highly significant ($P<0.01$) when total mean daily excretions were compared as well as when FGA values were related to dietary histidine intake. Injection of Vit. B_{12} decient chicks with 1 γ Vit. B_{12}/day has been reported to cause FGA excretion to decline to minimal levels after 2 days(4). The same effect has been observed when the diet of the Vit. B_{12} deficient chicks was supplemented with 0.1 mg Vit. B_{12}/kg of diet (unpublished data).

When Vit. B_{12} deficient chicks were given a DL-methionine supplement (incorporated in the diet at a level of 1%) for 24 hr, there was an immediate and marked drop in FGA excretion (Table II). On the day following methionine supplementation, FGA excretion rose to a level which was more than double the daily excretion during the pre-supplementation period. Then the amount of FGA dropped to the original level on the fifth day of the experiment. This pattern of FGA excretion is also shown in the photograph of the paper strips (Fig. 1) on which the com-

TABLE IV. Effect of Dietary Fat Level upon Excretion of FGA by Vit. B_{12} Deficient Chicks.*

Day	Dietary fat, %	Body wt, g	Histidine intake, mmole/day	μmole FGA Per day	μmole FGA Per mmole histidine
		Exp. 1 (ad libitum)			
1	4	180	2.6	65	25
2	4	190	2.5	87	35
3	24	195	1.7	103	61
4	24	200	1.9	108	57
		Exp. 2 (restricted food intake†)			
1	4	171	1.4	157	112
2	4	173	1.4	144	103
3	24	178	1.4	167	119
4	24	183	1.4	158	113

* 5 chicks in Exp. 1, 8 in Exp. 2; all 21 days old on day 1 of each experiment. All chicks received the low fat diet prior to 21 days of age.

† Amount of diet offered each chick each day was 75% of the avg amount consumed by the group on the day prior to day 1.

ponents in extracts of excreta were separated electrophoretically. These were from one chick during successive days of the above experiment. No formimino-containing compound other than FGA has been detected during this work by either the chromatographic or electrophoretic procedures described above.

The chicks ate more of the diet on the day when the methionine supplement was given (Table II). This increase may have influenced the excretion of FGA on the day following supplementation. Food intake on the day of methionine supplementation was therefore limited to the same amount of diet as that eaten on the day prior to the methionine supplement (Table III). In this experiment methionine did not lower FGA to as low a level as that seen in Table II. A possible explanation may lie in the fact that the chicks ate all of their diet before the end of the 24 hr period and part of the "post-methionine" high rate of FGA excretion may have been included in this day's excretion. However, the rise in FGA excretion on the day following supplementation was similar to that in Table II.

Several compounds biochemically related to methionine were tested for their effect upon FGA excretion (Table III). Each was incorporated into the diet for one day at a level equimolar to the 1% supplement of methionine. The first 2 days of each of these experiments serve as the basis of comparison for assessing the effect of each of the supplements. None of these compounds (DL-homocysteine thiolactone HCl, DL-homocystine, L-cystine, choline chloride, or betaine) reduced FGA excretion. In fact FGA excretion was slightly higher on the day that betaine was given. When either homocystine or choline chloride was given, there was a moderate increase in mean FGA excretion on the day following the supplement; however, these effects were small when compared with those of methionine.

Chicks raised to 3 weeks of age on the Vit. B_{12}-free diet that contained only 4% of fat excreted large amounts of FGA (Table IV). When the fat content of this diet was increased to 24% there was a moderate increase in FGA excretion. There was a decrease in the weight of food ingested when the fat content of the diet was increased, probably due chiefly to the higher caloric density of the high fat diet. A second experiment was conducted in which food intake was restricted during the initial 2-day FGA measurement period. It was thus possible to keep food intake constant during the time that both the low and the high fat diets were fed. In this experiment there was no change in FGA excretion when the dietary fat intake was increased.

Discussion. The dramatic effect of a methionine supplement in decreasing the high rate of FGA excretion by Vit. B_{12} deficient chicks confirms the findings that Silverman and Pitney obtained with the rat(1). The mechanism of this effect has been discussed (1,7); however, it is still largely a matter for conjecture. In these experiments only the intact methionine molecule was effective. The very rapid effect of the methionine supplement as compared to that of Vit. B_{12} suggests that methionine may be directly involved in the metabolism of the formimino group arising from dietary histidine. The marked increase in FGA excretion following removal of the methionine supplement apparently was not related to the increased food intake that occurred when additional methionine was incorporated in the diet (Table III). A relatively small proportion of the dietary histidine was excreted as FGA by the deficient chicks. It is therefore possible that the Vit. B_{12} deficient chicks had become adapted to use efficiently the small amount of available methionine for metabolism of the formimino group and that this efficiency was temporarily lost when the 24 hr high methionine supplementation period ended. This may account for the observed excretion pattern (Table II, Fig. 1).

FGA excretion of individual Vit. B_{12} deficient chicks has been found to be fairly constant from day to day; however, somewhat larger differences in control values have been found between different experiments (days 1 and 2, Table III). These experiments were carried out over a period of several months and variability in the chicks may have been a factor. The 2 experiments reported in Table IV were conducted almost

FIG. 1. Separation of amino acids by electrophoresis. The strip of each pair marked "A" was exposed to ammonia for 1 hr prior to staining with ninhydrin. The origin was marked with pencil on each side of each pair at the center of each strip. During electrophoresis the position of the lower ends of the strips as seen in the photograph were at the anode side. The patterns resulted from application of the following solutions: (1) standard FGA, 1 μmole/ml, (2) extract from vit. B_{12} deficient chick, 0.8 μmole FGA/ml (by colorimetric analysis), (3) same chick, day of 1% methionine supplement, 0.08 μmole FGA/ml, (4) same chick, first day after methionine supplement removed, 4.85 μmoles FGA/ml, (5) same chick, second day after methionine supplement removed, 0.9 μmole FGA/ml. Each day's excreta were made to a total volume of 50 ml.

successively. These data offer the suggestion that restriction of food intake may increase excretion of FGA. This possibility is being investigated further.

It is difficult to explain the differences in FGA excretion pattern between the 2 Vit. B_{12} deficient animal species and human subjects with pernicious anemia. Luhby *et al.* (8) and Spray and Witts(9) found that pernicious anemia patients excreted insignificant amounts of FGA after daily histidine loading tests of 15 and 2 g, respectively. In view of the results with animals, it is suggested that the methionine intake of the patients may have been sufficient to mask the effect of their Vit. B_{12} deficiencies upon FGA excretion. It is significant that a high level of dietary fat was not necessary to cause Vit. B_{12} deficient chicks to excrete large amounts of FGA. This is contrary to the earlier observations on growth of the chick fed this Vit. B_{12} deficient diet(3). The level of dietary fat is probably not involved in the differences between man and the chick.

Summary. Young chicks fed a diet deficient in Vit. B_{12} for 3-5 weeks excreted large amounts of formiminoglutamic acid (FGA) when compared to control chicks that received Vit. B_{12} in the diet. The high excretion rate of FGA by the Vit. B_{12} deficient chicks was reduced to very low levels by incorporation of 1% DL-methionine into the diet for 24 hr. This effect was not observed with supplements of DL-homocysteine thiolactone HCl, DL-homocystine, L-cystine, choline chloride, or betaine. Although a high level of dietary fat had previously been found necessary to cause poor growth in the absence of Vit. B_{12}, FGA excretion was quite high with either 4 or 24% of fat in the diet.

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Blood Volume and Aldosterone Secretion in Hypertension and Primary Aldosteronism.* (26736)

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Observation of an expanded extracellular fluid and plasma volume in 2 cases of primary aldosteronism(1) suggested that the hypervolemia might be a useful differential point in its diagnosis, particularly in distinguishing the hypertensive patient in whom the possibility of aldosteronism should be considered.

Case reports. Primary aldosteronism. L.B., a 39-year-old white female, and R.T., a 25-year-old white male, had known hypertension of 14 and 6 years duration respectively. Both had hypernatremic, hypokalemic alkalosis for about 6 years. On admission to hospital blood pressure in both was 220/130 mm Hg. Cardiomegaly, Keith-Wagner Grade II hypertensive retinopathy, and a positive Trousseau sign were observed in each. No edema or congestive heart failure was evident. The mean of 9 urinary aldosterone determinations during sodium intakes higher than 100 mEq was 29 μg/24 hours in L.B. (normal 3-15 μg/24 hours). Mean of 5 urinary aldosterone values in R.T. was 40 μg/24 hours. At operation an 8.4 g left adrenal adenoma in L.B. was removed and, in R.T., a 4.5 g right adrenal adenoma. Two months after excision serum electrolytes were normal in both patients and blood pressure had fallen to 140/90 mm Hg in L.B. and 170/100 in R.T.

Hypertension. Blood pressure, serum electrolytes and serum creatinine determinations are summarized in Table I. Blood volume and urinary aldosterone were determined while sodium intake was in excess of 100 mEq/24 hours. The selected patients had no evidence of congestive failure, edema, or propensity to retain sodium with salt loads. Patient J.M. is of particular interest since during our observation she developed malignant hypertension with papilledema and subsequently died of bacterial pneumonia. The adrenal glands were normal at postmortem examination. Patient M.S. also had malignant hypertension, which did not respond to antihypertensive medications. Postmortem study revealed normal adrenal glands and severe nephrosclerosis.

Methods. Red cell volume was determined by a modification of the $NaCr^{51}O^4$ method of Sterling and Gray(2,3). *Plasma volume* in the cases of primary aldosteronism was measured directly with T-1824(4) or I^{131} human serum albumin(5), thus allowing calculation of true whole blood volume and body hematocrit. Values for whole blood volume in the group of hypertensives (Table II) were calculated from red cell volume and venous hematocrits uncorrected for plasma trapping or for differences between the hematocrit of venous blood and that of the body as a whole. *Extracellular fluid* was determined by volume of dilution (Inulin)(7). Normal values were predicted at 16.2% of body weight. *Urinary aldosterone* was measured by the double isotope derivative technic of Kliman and Peterson(8) after hydrolysis at pH 1 for 24 hours at room temperature. Normal values with sodium intakes above 100 mEq/24 hours in this laboratory are 3-15 μg/24 hours.

Results. Primary aldosteronism (Table II). Preoperative. In patients L.B. and R.T., respectively, a marked expansion of extracellular fluid of 58% and 57%, total

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TABLE I. Blood Volume and Urinary Aldosterone in Hypertension.

Patient, age & sex	Blood pressure, mm Hg	Serum electrolytes Na	K	Cl	CO_2	Serum creatinine, mg/100 ml	Red cell volume Hct., %	Pred., l	Obs., l	Dev., %	Total blood vol (Cr^{51})* Pred., l	Obs., l	Dev., %	Urinary aldosterone, μg/24 hr
		meq/l												
P.C. 36 ♂	235/170	138	3.5	93.5	29.4	1.9	41.9	1.60	1.27	79	3.40	3.02	89	3.4
R.P. 50 ♂	200/140	145	3.7	99.9	31.4	2.1	39.9	2.53	2.20	87	5.27	5.52	105	3.1
C.D. 37 ♀	160/110	143	4.1	106.0	23.9	1.4	30.0	1.82	.93	51	4.00	3.10	78	13.0
V.M. 56 ♀	220/120	132	3.7	85.0	28.2	2.8								7.7, 9.4
D.C. 6 ♀	170/125	132	4.6	100.0	29.6	1.4								6.4
J.M. 36 ♀	170/120	138	3.1	77.5	33.0	3.7	37.3	1.73	.93	54	3.63	2.50	69	5.4
J.M.† 36 ♀	210/130	118	5.5	79.1	21.1	10.0	30.3		1.09	61	3.63	3.56‡	98	36.0
M.S.† 4 ♀	260/160	146	4.6	113.0	20.6	1.5								28.6

* Predictions based on height and weight according to Wennesland *et al.*(6).
† Malignant phase.
‡ Given 2 units of blood and 250 ml 3% saline day before volume measurement. Urinary aldosterone determined 5 days before blood volume.

TABLE II. Blood Volume Alteration in Primary Aldosteronism.

	Height, cm	Wt, kg	Plasma vol l	%*	Red cell vol (Cr^{51}) l	%*	Total blood vol l	%*	Body hct.: Venous hct.	ECF (inulin) l	%*	Hct., %	Urinary aldosterone, μg/24 hr
L.B., preoperative	165.5	50.2	3.48 (T-1824)	(176)	1.58	(113)	5.06	(145)	.89	13.0	(158)	35.0	29
L.B., postoperative			2.75 (T-1824)	(140)	1.20	(73)	3.95	(111)	.99	10.6	(124)	30.7	5
R.T., preoperative	189.5	75.2	4.37 (I^{131})	(148)	2.22	(99)	6.59	(129)	.94	19.0	(157)	35.8	40
R.T., postoperative			2.94 (I^{131})	(100)	2.14	(97)	5.08	(100)	.91	12.0	(100)	46.1	3

* % of predicted values.

blood volume 45% and 29%, and plasma volume 75% and 48% above predicted normal but with low hematocrits were noted. Serum albumin concentration was 3.7 g/100 ml in L.B. and 4.4 g/100 ml in R.T. Red cell volumes in both patients were *normal*. *Postoperative*. Eight weeks after operation all these measurements had been restored to normal in R.T. In L.B., the restoration was toward normal; total blood volume was but 11% above normal. Postoperative serum albumin concentration was 3.6 g/100 ml in L.B. and 4.6 in R.T. *Hypertensive patients* (*Table I*). Similar measurements performed in 4 patients with essential and in one with malignant hypertension revealed normal-to-decreased total blood volumes. Red cell volume was decreased 51-87% of predicted normal values. However, urinary aldosterone levels had increased to 28.6 (M.S.) and 36.0 (J.M.) in the patients with malignant hypertension, while still normal in the group with essential hypertension (3.1 to 13.0 μg/24 hours).

Discussion. In the 2 cases of primary aldosteronism it appears that the expanded plasma volume is a consequence of an increase in extracellular fluid produced by chronic secretion of excessive aldosterone similar to that seen after continued administration of desoxycorticosterone acetate(9) or aldosterone(10). Red cell volume was normal, and the plasma expansion was thus revealed by the low hematocrit in both cases. Serum albumin concentration in both patients was normal and remained unchanged after removal of the adenomas. Colloid osmotic pressure was strikingly unaltered irrespective of changes in aldosterone secretion. Complete restoration of total blood volume, plasma volume and extracellular fluid without changes in red cell volume occurred in R.T. 2 months after operation. In L.B., total blood volume approached normal values with a 46% fall in plasma volume. Significant anemia resulting from blood loss at operation may have contributed, in part, to the still expanded plasma volume.

In contrast, total blood volume and urinary aldosterone levels in 4 cases of severe essential hypertension were normal or even depressed. Total blood volume and extracellular fluid have been found previously to be normal in hypertension(11,12). Grollman (13) observed increases in extracellular fluid but normal plasma volumes in hypertension. Urinary aldosterone assays in essential hypertension have been found normal(14). Only in malignant hypertension has increased aldosterone secretion been clearly established (15). In one case of transition from essential to malignant hypertension without occurrence of heart failure, urinary aldosterone increased greatly without associated increases in blood volume or changes in plasma electrolytes. The adrenals were not enlarged at the time of postmortem examination. The blood volume in the second case of malignant hypertension was not determined, but urinary aldosterone was increased and there was no indication of hypervolemia, as reflected by a normal hematocrit without serial change and the absence of congestive heart failure.

Thus the demonstration of an expanded plasma volume, which may be indicated by a low hematocrit in a hypertensive patient with hypernatremia and hypokalemia, suggests primary aldosteronism. The hypertensive patients studied also showed a slight decrease in hematocrit, which reflected a true red cell volume deficit and not an expanded plasma volume, as seen in the 2 cases of primary aldosteronism. The differentiation of primary aldosteronism from malignant hypertension might be clarified further by a normal blood volume.

Summary. Two cases of primary aldosteronism were demonstrated to have expanded extracellular fluid, total blood volume, and plasma volume, with restoration toward normal after removal of adrenocortical adenomas. Red cell volumes were normal in both. No increase in urinary aldosterone was observed in 5 patients with essential hypertension. Blood volume was normal or slightly decreased. Increased urinary aldosterone without hypervolemia was observed in 2 patients with malignant hypertension. Thus increased total blood volume in hypertensive patients, due solely to plasma volume and anemia (low hematocrit), should prove a

useful addition to the diagnostic screen for primary aldosteronism.

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Activation of Tremorine by Liver.*† (26737)

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Tremorine (1,4-dipyrollidino-2-butyne) was reported by Everett(1) to produce sustained tremors and parasympathetic-like stimulation in several species of laboratory animals. Since it elicits tremors and other parkinsonian-like signs, Tremorine has been used in routine screening for potential anti-parkisonian drugs. The report(2) that an hour elapses before the cat shows typical drug effects after administration of Tremorine (10-20 mg/kg) suggested that Tremorine may have to be biotransformed before it becomes pharmacologically active. Evidence presented here indicates that the liver converts Tremorine to an active form which has pharmacologic properties differing from those of the original product.

Materials and methods. Young adult male Swiss-Webster mice, hamsters weighing 100-150 g, 2-3 kg cats and 5-10 kg dogs were used. Tremorine dihydrochloride‡ was dissolved in pH 7.4 Krebs-phosphate buffer (2 mg/ml) prior to incubation with liver. Homogenates and tissue slices totaling 2-4 g wet weight from the livers of mice, hamsters, or rats were incubated at 37°C for 2 hours in a shaking incubator with 12 ml of buffered Tremorine solution in 250 ml flasks (O_2 gas phase). After incubation filtrates of the solution were injected into animals in doses expressed as equivalents of Tremorine present before incubation.

Time of onset of tremors was measured from injection to time of head and limb tremors of a fully developed intensity as judged by a single observer throughout the study. Five or more mice per group were used in each experiment, except as noted.

Results. Tremorine incubated with mouse liver slices produced tremors within 5 sec after intravenous injection into mice, whereas non-incubated Tremorine required more than 15 min to produce effects (Table I). It

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TABLE I. Effect of Incubation of Tremorine with Liver Slices and Route of Injection on Time of Onset of Tremors in Mice.

Conditions of incubation	SKF-525A* pretreatment, mg/kg	Tremorine inj. route†	No. of animals	Onset of tremors ± S.D.
Not incubated	0	Intraper.	12	6.3 ± 2.0 min.
Idem	0	Intrav.	15	16.3 ± 3.0 "
"	0	Subcut.	6	20.2 ± 4.1 "
"	25	Intrav.	6	None in 5 hr
Incubated without drug	0	"	6	No effects
Incubated with drug	25	"	25	Within 5 sec.
Idem	0	"	6	*Idem*

* Injected intraper. 1 hr before Tremorine.
† Injected dose equivalent to 20 mg/kg Tremorine dihydrochloride.

was further noted that intraperitoneal administration of non-incubated Tremorine produced tremors sooner than intravenous or subcutaneous administration (Table I), suggesting that the liver activates the drug more rapidly after intraperitoneal injection. More rapid and shorter-lasting effects (1½ hr duration) were produced by 5 mg/kg of the activated product than by 20 mg/kg of non-incubated Tremorine (3-4 hr duration), although the 2 products evoked similar effects of approximately equal intensity. In 3 cats activated Tremorine produced vomiting, salivation, agitation, and hissing beginning 5 min following intraperitoneal injection of a dose equivalent to 8 mg/kg. Non-activated Tremorine preparations did not begin to produce these effects for 15-30 min following doses of 5 and 10 mg/kg, as seen in 4 cats.

SKF-525A (diethylaminoethyldiphenylpropylacetate), an inhibitor of liver microsomal activity(3), given to mice in a dose of 25 mg/kg 30 min prior to Tremorine (20 mg/kg) completely prevented the onset of tremors. Unlike atropine(4) SKF-525A did not abolish tremors already established. Liver-activated Tremorine, however, produced tremors within 5 sec following intravenous administration, whether or not the mice were pretreated with SKF-525A (Table I). Control solutions in which liver was incubated without Tremorine produced no effects in mice.

Two other reported inhibitors of liver microsomal activity(5) were also tested in doses of 25 mg/kg, as prophylactic agents against Tremorine in mice. Lilly 18947 (2,6-dichloro-6-phenylphenoxyethyldiethylamine hydrobromide) prevented the onset of tremors, but iproniazid did not. Four antihistamines (diphenhydramine, tripelennamine, pyrilamine, doxylamine) structurally related to SKF-525A did not prevent tremor development in mice when given at 25 mg/kg one hour prior to 20 mg/kg of Tremorine.

Although SKF-525A in doses of 25 mg/kg or higher, did not completely block the effects of large doses of Tremorine (80 mg/kg) in mice, the tremors and other signs were delayed in onset. SKF-525A also delayed the onset of tremors in rats given 10 mg/kg Tremorine.

The effect of Tremorine and its activated product on blood pressure was studied in 5 dogs anesthetized with pentobarbital. Intravenous injection of 100 μg/kg of the activated product produced bradycardia and an immediate fall in blood pressure of approximately 65 mm Hg, which was restored to control level within 65 sec after injection. Doses of the activated product as small as 1 μg/kg produced a significant fall in blood pressure of 8-10 mm Hg. Atropine (0.5 mg/kg) abolished these responses. Non-incubated Tremorine in doses up to 1 mg/kg had no observable effect on blood pressure and at 5 mg/kg the immediate effect varied from none to a slight fall of 5-10 mm Hg, although a gradual decline of approximately 50 mm Hg occurred over a period of about 1½ hours. This depressed blood pressure returned to normal after atropine (0.5 mg/kg).

Diarrhea is recognized as a common sign of the widespread cholinergic effects produced by Tremorine. Tremorine, however, in a concentration of 3.5×10^{-5}M had no

effect on the activity, as recorded kymographically, of strips of rabbit intestine suspended in Tyrode's solution, and at a level of 7×10^{-5}M even slightly depressed it. By contrast, activated Tremorine at a concentration equivalent to 3.5×10^{-5}M produced a marked stimulation of the rabbit intestinal strip, an effect antagonized by atropine (1:10,000,000). Interestingly, the activity of intestinal strips stimulated by activated Tremorine (3.5×10^{-5}M) or acetylcholine (1:10,000,000) returned immediately to a normal pendular movement when non-incubated Tremorine (1.4×10^{-4}M) was added.

Rat and hamster liver homogenates and hamster liver slices incubated with Tremorine also activated Tremorine, since filtrates from these preparations consistently produced typical symptoms within 5 sec in 20 mice injected intravenously with doses equivalent to 20 mg/kg of Tremorine. Urine was expressed from the bladders of 30 mice for 3 hr starting 15 min after they received 20 mg/kg of Tremorine. This urine, diluted with an equal volume of saline and injected intravenously into 8 mice in a dose of 0.1 ml, produced immediate signs of Tremorine poisoning of an intensity similar to that seen in the mice from which the urine was collected, but of shorter duration. Normal urine, similarly tested, had no effect.

The incubates of Tremorine with liver slices from hamsters, rats and mice, as well as urine from Tremorine-treated rats and mice were extracted with chloroform after adjusting to pH 11. The oily residue remaining after evaporating the combined extracts to dryness was dissolved in 0.01 N HCl, and tested for activity in normal and SKF-525A-treated (25 mg/kg) mice, on the blood pressure of 4 dogs anesthetized with pentobarbital and on the isolated rabbit intestine. It was found that 3 mg/kg of any of these residues when injected into mice intravenously immediately produced severe symptoms characteristic of Tremorine. Lower doses produced effects of variable duration and intensity, the residues from urine appearing to be more potent than those from liver slices.

The extracts from the various liver preparations also produced an immediate transient fall in the blood pressure of anesthetized dogs, ranging from 45 to 70 mm Hg when 100 μg/kg of any residue was injected intravenously, the response apparently varying with the efficiency of activation in different incubates. Extracts from the urine of Tremorine-treated rats and mice were more potent in depressing blood pressure, a fall of approximately 65 mm Hg resulting from 50 μg/kg.

Extracts of urine from Tremorine-treated rats and mice as well as Tremorine liver slice incubates from hamsters, rats and mice all produced a sustained tonic contraction of the isolated rabbit intestine when added to the bath to give a final concentration of 1 μg/ml in case of urine extract and a concentration equivalent to 3.5×10^{-5}M Tremorine in case of the liver incubates. Control liver incubates and extract residues produced no effect in mice, on blood pressure of dogs, or on the isolated intestine of rabbits.

Discussion. The above data indicate that the liver converts administered Tremorine to an active form having pharmacologic properties that sharply distinguish it from its original form. The effects of activated Tremorine in stimulating rabbit gut *in vitro* and in momentarily depressing blood pressure of the anesthetized dog are consistent with the diarrhea, bradycardia, prolonged depression of blood pressure and other cholinergic effects seen in the fully developed picture of Tremorine's action. Before it is activated, however, Tremorine does not stimulate the isolated rabbit gut, and it lowers the dog blood pressure only after an appreciable latent period. The rapidity with which activated Tremorine acts suggests that the characteristic delay in onset of action of Tremorine in most animals is the time required for the liver to activate it. Since Tremorine is presumably activated continuously as long as it persists in the body, it would be expected that the effects of administered Tremorine should last longer than those produced by single acute doses of the active form. Thus, the prolonged depression of blood pressure gradually induced by Tremorine over a period of 90 min is markedly dif-

ferent from the sharp evanescent fall in blood pressure produced by intravenous injection of activated Tremorine.

The fact that SKF-525A, an inhibitor of liver microsomal activity, blocks Tremorine but does not block activated Tremorine serves as a basis for determining the presence of the active form. It points, furthermore, to the liver microsomes as the site of the activation process.

Although the pharmacologic properties of activated Tremorine from different sources (urine of Tremorine-treated mice and rats, and incubates of Tremorine with preparations of liver from different species) are similar, it is clear that their chemical identity has not been established. Studies to determine the chemical nature of activated Tremorine are in progress.

Summary. Mouse liver slices converted Tremorine to an active form which differed from non-incubated Tremorine preparations (a) by its faster onset of action in untreated mice and cats, (b) by not being blocked by SKF-525A (diethylaminodiphenylpropylacetate), an inhibitor of liver microsomal activity, (c) by stimulating rabbit gut *in vitro* and (d) by producing an immediate slowing of the heart and fall in blood pressure on intravenous injection into anesthetized dogs. Material having the biological properties of activated Tremorine was also found in the urine of rats and mice given Tremorine and in solutions of Tremorine incubated with hamster liver slices and with hamster and rat liver homogenates.

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Tissue and Serum Manganese Levels in Evaluation of Heart Muscle Damage. A Comparison with SGOT. (26738)

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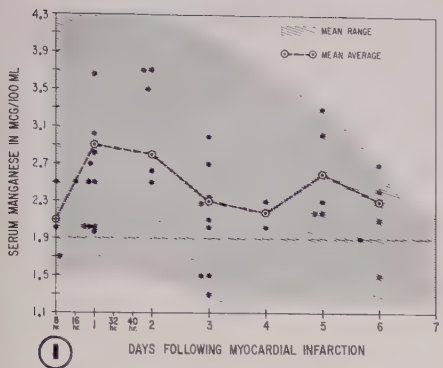
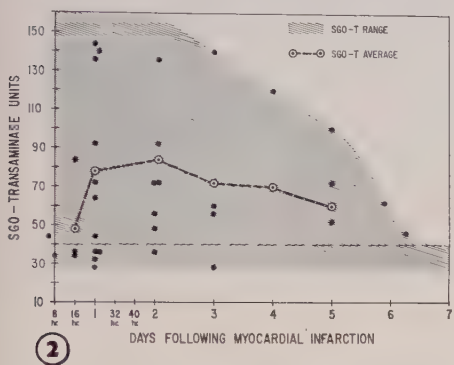
The normal myocardium contains about 1800 μg of manganese per 100 g of dried tissue; this is roughly 2,000 times the manganese concentration present in the blood serum. Myocardial injury results in a sharp decrease in tissue manganese associated with a proportional rise in serum manganese content. Following myocardial infarction tissue manganese *decreases* to approximately 30% of its original value. Concomitantly, serum manganese *increases* approximately 2½ times. Thus, after infarction human serum contains more than 2.0 μg manganese per 100 ml, whereas prior to infarction, serum manganese content is about 1.0 μg manganese per 100 ml.

Because tissue manganese is lost very rapidly from the infarcted myocardium following cardiac injury, and a reciprocal rise in manganese concentration is observed in the serum, determination of serum manganese level should be of great value in detection of myocardial injury and, in equivocal cases, in confirmation of acute myocardial damage.

In a study at Los Angeles County Hospital, serial measures of serum manganese levels in 26 patients with unequivocal evidence of myocardial infarction were compared with serum samples from 25 patients of comparable age, sex and race admitted to the hospital with complaints other than myocardial injury. This report correlates clinical findings in our patients with serum manganese levels, as determined spectrographically from 80 blood samples taken from the patients with acute myocardial infarction, and





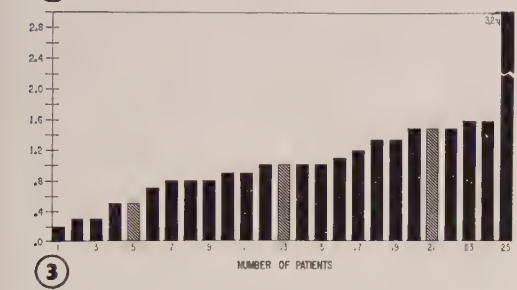


FIG. 1. Serum manganese level in myocardial infarction.

FIG. 2. Serum glutamic oxalacetic transaminase (SGOT) in myocardial infarction.

FIG. 3. Serum manganese level in conditions other than myocardial infarction. Barred columns indicate cases in which SGOT was elevated, giving false-positive results.

38 samples from the control patients.

In our patients the first sample of blood serum is received within 12 to 48 hours after onset of acute myocardial infarction. Samples also are obtained on the third and fifth day of the acute episode and, in a few instances, an additional sample is collected on the seventh day.

Blood samples are refrigerated until subjected to spectrographic analysis. Serum is separated from red blood cells before refrigeration to prevent gross hemolysis.

Within 8 to 12 hours following myocardial injury, manganese level rises; a maximum is reached within 24 to 48 hours. Serum manganese level remains elevated for 5 or 6 days, after which manganese level gradually falls to normal. Height and duration of serum manganese elevation are roughly proportional to amount of myocardial necrosis as estimated on the basis of ECG evidence. Although a close correlation exists between serum manganese level and SGOT value, serum manganese concentration is far less likely to give false positive values.

Fig. 1 depicts serum manganese values obtained in 26 patients with myocardial infarction. The shaded portion outlines the range of values. Solid dots represent actual values obtained; larger circles connected by broken lines indicate average values. In all instances but one, serum manganese level rose to above 2.0 μg/100 ml within 48 hours after onset of chest pain.

Secondary elevations in serum manganese level, noted in several instances following recurrent chest pain, apparently indicated extension of an existing infarction or development of a fresh infarction.

Determinations of SGOT values obtained simultaneously with our serum manganese values are depicted in Fig. 2. Note the similarity of ranges of values in the 2 measures; the averages, too, are rather similar in nature; however, myocardial injury is detected earlier in determinations of serum manganese, with the level rising indicatively within 8 hours of onset of pain, and remaining at elevated levels for longer periods than the serum GOT.

In 24 of the 25 patients in our control series, serum manganese levels were well below 1.9 μg/100 ml. Simultaneously determined SGOT levels in 3 of these patients were elevated and suggestive of myocardial injury; however, the low serum manganese levels in the 3 patients afford definite evidence that acute myocardial injury had not occurred.

Fig. 3 depicts the height of serum manganese in the 25 control patients in our series. The barred columns represent 3 pa-

TABLE I. Comparison of Tissue* and Serum Metal Concentrations† of Patients with and without Myocardial Infarction.

	Tissue metal conc. in mg/100 g dried tissue		Serum metal conc. in μg/100 ml	
	Acute M.I. (33 determinations)	Without M.I. (100 determinations)	Acute M.I. (38 determinations)	Without M.I. (80 determinations)
Metal	Mean value ± S.E.	Mean value ± S.E.	Mean value	Mean value
Aluminum	1.770 ± .009	3.270 ± .237	4.30	3.70
Calcium	29.336 ± 6.451	44.800 ± 5.390	101,000.00	99,000.00
Manganese	.072 ± .007	.180 ± .011	2.42	1.01

* Tissues obtained at autopsy.

† Mean values.

tients (Nos. 5, 13, and 21) with infectious hepatitis, hepatoma and cholecystitis, respectively, whose SGOT level was above 40 units and therefore consistent with myocardial injury. Low serum manganese levels in these patients definitely exclude the possibility of acute myocardial injury.

In the 25th patient in our control series, serum manganese concentration was 3.2 μg/100 ml on the first postoperative day, within the range usually indicative of myocardial infarction. This patient had undergone laparotomy, gastrostomy, and had had a liver biopsy the previous day. Apparently, following extensive major surgery, elevation in serum manganese concentration may mimic levels occurring after myocardial injury. Therefore, although a manganese level within normal limits definitely precludes the presence of acute myocardial injury, an elevated manganese level in the postoperative patient cannot be accepted as evidence of acute heart muscle injury.

In Table I, tissue aluminum, calcium, and manganese values obtained postmortem from "normal" patients (*i.e.*, without known cardiovascular disease) and patients with myocardial infarction are compared with serum concentrations of "normal" patients and patients with fresh myocardial infarcts. Note that concentration of all metals is lowered in tissues of the patient with myocardial infarction, and a reciprocal rise is observed in blood serum. However, the alterations in manganese are much more pronounced both in tissue and serum.

In addition to blood samples from patients with myocardial infarction and control series, serum samples from 3 patients with old myocardial infarcts, 4 patients with angina pectoris or coronary insufficiency, and 4 patients with pulmonary infarction were subjected to spectrographic analysis.

Angina Pectoris, Coronary Insufficiency, or Slight Myocardial Damage. Ordinarily, angina pectoris does not cause an elevation in serum manganese level. Nevertheless, in our series, 2 patients whose ECGs were not suggestive of acute myocardial infarction had elevations of serum manganese. The first of these patients had suffered a myocardial infarction 10 years previously. At hospitalization, she complained of severe chest pain of 5 days' duration. An SGOT value of 65 units was obtained at this time; her serum manganese value was 2.5 μg/100 ml. An SGOT determination on the second day of hospitalization, the sixth day of chest pain, was 113 units, which was accepted as definite evidence that a transmural myocardial infarction had occurred, and treatment for acute myocardial infarction was instituted despite lack of corroborative ECG evidence.

The second patient had for 12 years had angina pectoris relieved by nitroglycerin. She was admitted to the hospital because of crushing chest pain which radiated down the left arm and was not relieved by nitroglycerin. Although ECG confirmation of acute myocardial infarction was absent, the patient was treated for acute myocardial infarction. The SGOT value rose gradually until, on the fourth hospital day, it reached 60 units. A serum manganese determination also obtained on the fourth day showed 2.2 μg manganese per 100 ml. By the fifth day, serum manganese value had dropped to 1.5 μg/100 ml. Both erythrocyte sedimentation rate and white blood count were elevated.

Old myocardial infarctions. One patient

had suffered myocardial infarction 4 months previously. Although she complained of chest pain, her ECG showed no indication of acute infarction and her SGOT value was 58 units. Serum manganese determinations upon 3 separate occasions during her hospitalization all showed less than 1.5 μg of manganese per 100 ml of serum, suggesting absence of myocardial infarction.

Pulmonary infarction. Serum manganese levels of 3 of the 4 patients with pulmonary infarction were within normal limits. A serum manganese level compatible with myocardial infarction occurred in one patient, a 45-year-old man who complained of severe chest pain which radiated to the neck and left shoulder. This patient, who had suffered a transmural myocardial infarction 18 months previously, entered the hospital with pulmonary edema. An ECG taken at time of hospital entry was indicative of acute cor pulmonale with severe right atrial and right ventricular hypertrophy. Serial determinations of SGOT and serum manganese are available for this patient: Blood specimens drawn on the first day showed an SGOT value of 4400 units and a serum manganese value of 3.5 μg/100 ml. On the second day of hospitalization, serum manganese level was 4.5 μg/100 ml. SGOT levels obtained on third and fourth day of hospitalization yielded values of 4400 and 2250 units, respectively. On the sixth day of hospitalization, SGOT concentration was 335 units. At autopsy the patient was found to have suffered massive pulmonary infarction.

We have found the serum aluminum value useful in differential diagnosis of pulmonary infarction and myocardial infarction. Following pulmonary infarction, the tissue aluminum value of the lung is lowered, and a 4-fold increase occurs in serum aluminum value. A comparably elevated serum aluminum value has not been noted in any condition other than pulmonary infarction. Additional information furnished by the serum aluminum value corroborates the diagnosis in pulmonary infarction and helps to differentiate between pulmonary and myocardial infarction. From our limited evidence it appears that a very high serum manganese value, above that ordinarily occurring in acute myocardial infarction, may indicate presence of massive pulmonary infarction, and serum aluminum value should be used as a check.

Summary. Whereas, in our series, serum manganese values in patients with congenital, infectious, degenerative, or neoplastic disease were below 1.9 μg/100 ml of serum, serum manganese was elevated above 2.0 μg following acute myocardial infarction in every instance. Massive pulmonary infarction, which also may be accompanied by high serum manganese levels, can be differentiated from myocardial infarction by additional information supplied by serum aluminum values, which are elevated following pulmonary injury but not following myocardial damage. In patients with myocardial infarction, height of the serum manganese level provides an index to extent and severity of cardiac damage as estimated on the basis of SGOT level and the ECG. Although a close correlation exists between SGOT and serum manganese values, our data indicate that serum manganese concentrations are less likely to yield false positive indications of myocardial damage.

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Metabolism of Adenosine by the Isolated Anoxic Cat Heart.* (26739)

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In extracts of heart muscle the most active pathway of nucleotide synthesis is *via* direct phosphorylation of adenosine(1). In support of these observations, experiments on the isolated intact perfused cat heart have demonstrated a rapid incorporation of labelled adenosine into cardiac nucleotides in the presence of oxygen(2). The present study indicates that adenosine 8-C^{14} is also incorporated into myocardial nucleotides in the absence of oxygen.

Methods. Cat hearts were isolated and perfused with Tyrode's solution as described previously(2) and aerobic control collections of 200 ml of perfusate were made. The hearts were then perfused with Tyrode's solution equilibrated with a gas mixture of 95% nitrogen and 5% carbon dioxide for about 2 minutes before adenosine 8-C^{14} was added to the perfusion fluid. Adenosine was added in concentrations of 3.8×10^{-3} to 4.2×10^{-3} μM per ml, and the hearts were perfused with 200 ml of the solution. After a single passage through the heart the perfusates were collected in flasks immersed in ice.

The neutralized perchloric acid extracts of the hearts were analyzed for nucleic acid derivatives by a modification of the method of Cohn and Carter(3). The nucleotides were adsorbed on Dowex-1 resin, the columns were washed with 0.1 M ammonium chloride in 0.1 M ammonium hydroxide, and the nucleotides were eluted with 0.1 N hydrochloric acid in one fraction. The counts per minute in wash and eluate were determined. Nucleotide derivatives recovered in the perfusates were separated by paper chromatography, assayed enzymatically and counted. Details of the analytical procedures have been previously described(2).

Results. Perfusion of the cat heart with deoxygenated Tyrode's solution containing adenosine 8-C^{14} resulted in extensive conversion of the adenosine to myocardial adenine nucleotides during a single passage of the solution through the heart (Table I). In Experiment 1, 44% of the recovered counts were found in the acid soluble supernatant of the heart homogenate, whereas in Exp. 2 and 3 (Table I) about 54% of the recovered counts were present in this fraction. The balance of the recovered counts were present in the perfusates. A loss of 16-23% of the total counts occurred during the experimental procedures (Table I), and a maximum of 2% of the counts in the heart homogenates were found in the non-nucleotide eluates from the columns.

Characterization of the nucleotides in the 0.1 N hydrochloric acid column eluates by paper chromatography indicated that the greatest amounts of radioactivity, determined with a paper strip counter, were in the ATP, ADP, and AMP regions. Further characterization of the nucleotides in the 0.1 N hydrochloric acid eluates by paper chromatography of their acid hydrolysis products indicated that at least 85 to 90% of the radioactivity was in the adenine region. A small peak was observed in the ITP region of one of the hydrochloric acid column eluates. Some activity was also observed at the solvent front, suggesting the presence of adenine.

Analysis of the perfusates revealed the presence of most of the radioactivity in the inosine and hypoxanthine regions of paper chromatograms in all 3 experiments. A small radioactive peak was seen in the adenosine region in Exp. 3, and a small peak was also observed at the origin in this experiment, although no ultraviolet absorbing spot was visible.

It was previously observed(2) that enzy-

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TABLE I. Distribution of Perfused Adenosine 8-C^{14} between Myocardium and Perfusate.

Exp. No.	Adenosine 8-C^{14} perfused		CPM recovered		% of CPM recovered	Heart wt, g
	CPM	μM	Perfusate	Heart		
1	186,000	.84	88,000	68,000	84	12.3
2	182,000	.77	64,000	77,000	77	18.4
3	176,000	.76	69,000	76,000	82	19.9

matic assay detected no striking differences between amounts of inosine and hypoxanthine recovered during control and experimental periods when low concentrations of adenosine were perfused in the presence of oxygen. Similarly, no difference was observed in amounts of inosine and hypoxanthine recovered during the period of anoxia when adenosine was perfused. However, it should be emphasized that prolonged perfusion of anoxic Tyrode's solution (by recirculation) does produce significant increases in amount of inosine and hypoxanthine recovered in the perfusates compared to amount in oxygenated control perfusates. In Exp. 1 the perfusate contained a larger part of the radioactivity than the myocardium, in contrast to Exps. 2 and 3 (Table I). The heart stopped beating for about one minute during the perfusion period in Exp. 1, indicating severe anoxia. In the other 2 experiments the rhythm became irregular for varying periods of time but the heart beat was maintained.

Anoxia or perfusion with adenosine (4×10^{-3} μM/ml) produces coronary vasodilation. However, addition of adenosine to anoxic Tyrode's solution did not induce a greater coronary flow than that observed with anoxia alone.

Discussion. The average incorporation of adenosine 8-C^{14} into adenine nucleotides in the anoxic heart (51%) compares favorably with that observed when the adenosine is perfused in oxygenated Tyrode's solution (57%)(2). Since knowledge of the rate limiting step in incorporation of adenosine into cardiac nucleotides and the size and nature of precursor pools are not available in the present study, the reason for similar degrees of incorporation in presence or absence of oxygen is not apparent. It is possible that in the non-working heart energy derived from glycolysis is sufficient to maintain totle cardiac adenine nucleotides at normal levels and perhaps even maintain normal ratios of ATP, ADP, and AMP. The glucose present in the perfusion medium would be made more readily available under the conditions of the experiments since anoxia enhances glucose transport across the myocardial cell membrane and rate of phosphorylation in the isolated heart(4). The extent and rapidity of incorporation of the labelled adenosine into adenine nucleotides suggest active synthesis or turnover of myocardial nucleotides.

Summary. The anoxic isolated perfused cat heart incorporates approximately 50% of labelled adenosine into myocardial nucleotides with one passage of the adenosine-enriched perfusion fluid through the heart.

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Incorporation of P^{32} into Phosphatido-Peptide Fraction of Normal and Neoplastic Mouse Epidermis.* (26740)

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In 1959, Huggins and Cohn(1) reported studies on a phosphatido-peptide fraction from renal cortical tissue which was found to contain inositol, phosphorus, fatty acids, glycerol and amino acids presumably in peptide linkage. They also reported that this fraction exhibited a higher turnover rate in renal tissue when compared with phospholipid as measured by P^{32}. Distribution studies revealed that phosphatido-peptide was present in all normal tissues as well as in Ehrlich ascites tumor cells. These observations led us to compare the metabolism of P^{32} in phosphatido-peptide and other phosphorus-containing fractions between normal and tumor tissue. Consequently, the turnover rate of P^{32} in these fractions was determined *in vitro* using normal epidermis, "normal" epidermis from tumor-bearing mice and neoplastic epidermal tissue from mice.

Materials and methods. Male adult C57Bl/6 mice used in this study received laboratory chow and water *ad libitum*. The tumor, squamous cell carcinoma, was induced by topical painting with 20-methylcholanthrene to the dorsal unepilated interscapular skin daily for a period of 9 weeks as described previously(2). Histologically, the tumor showed the structure of an epidermoid carcinoma with occasional differentiation into spinous cells, keratinization and formation of epithelial pearls. Papillomas appeared within 9 weeks and squamous cell carcinoma about 12 weeks following the last application. Normal epidermis and "normal" epidermis from tumor bearing mice were obtained from the ventral surface of the mouse. Epidermal cells were harvested by scraping the sheets of shaved skin which had been incubated previously with 0.02% ethylenediamine tetracetic acid (EDTA)(3) for one hour at 0-4°C. Tumor cells were obtained after mincing the tumor mass with scissors and incubating with 0.02% EDTA for one hour at 0-4°C. Necrotic areas were removed before mincing the tumor. Tissues were incubated in Yeast extract-Eagle medium-Lactalbumin hydrolysate-Peptone medium (YELP)(4) under aerobic conditions at 37°C in Warburg vessels. Each ml of incubation medium contained approximately 50 mg wet weight of tissue and 10 μC of P^{32}. After 2 hours' incubation, the tissue was separated into the different phosphorus-containing fractions as described by Huggins and Cohn(1). The source of P^{32} and methods for determination of its activity were reported previously(1). The phosphatido-peptide content is expressed as μg P per unit dry weight and also per average cell because the latter may be altered in the case of the 3 different types of tissues studied. Each experiment represents the pooled tissues, epidermis or tumor, from 6 mice. The data in Table I and Fig. 1 represent the average of 4 experiments.

Results. Table I compares total phosphorus and P^{32} incorporation into phosphatido-peptide for normal epidermis, "normal" epidermis from tumor-bearing mice and neoplastic epidermal tissue. There was no apparent change in phosphatido-peptide phosphorus content of the tissue tested, in contrast to the marked stimulation of turnover of the phosphoryl radical as evidenced by the higher levels of specific activity both in neoplastic tissue and "normal" epidermis from the tumor-bearing mice when compared with normal epidermis.

Fig. 1 shows that not only is the specific

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TABLE I. Incorporation of P^{32} into Phosphatido-Peptides of Normal and Neoplastic Epidermis of Mouse Skin under *In Vitro* Conditions.

Tissue	Phosphatido-peptide content		Specific activity, cpm/μg P
	μg P/100 mg dry wt	μg P/cell $\times 10^{-7}$	
Normal epidermis	16.5	3.9	56
Epidermis from tumor-bearing mice	15	4.3	2511
Neoplastic epidermis (squamous-cell carcinoma)	10	4.2	2460

activity of the phosphatido-peptide phosphorus elevated in neoplastic epidermis and "normal" epidermis from tumor-bearing animals but also that these 2 tissues show a similar pattern for phospholipid, phosphoprotein and nucleic acid fractions. Of special interest, the specific activity of the nucleic acid phosphorus is higher in the "normal" epidermis from tumor-bearing mice after 2 hours of incubation than that found in the tumor. A similar pattern of incorporation of P^{32} *in vivo* was found in these 3 tissues; however, the differences between normal epidermis and the other 2 tissues were not nearly so great. A more detailed report including studies on the nucleic acid, phospholipid and phosphoprotein fractions of these tissues is in preparation.

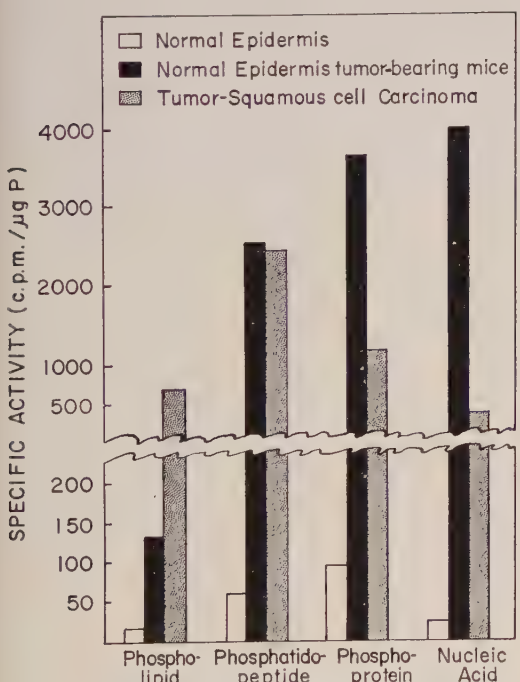

FIG. 1. Incorporation of P^{32} *in vitro* into phospholipid, phosphatido-peptide, phosphoprotein and nucleic acid fractions of normal epidermis, "normal" epidermis from tumor-bearing mice and neoplastic mouse epidermis. Specific activity is expressed as cpm/μg P. Aerobic incubation was for 2 hr in a medium containing about 30 mg wet wt of tissue and 10 μC of P^{32}/ml.

Discussion. In an investigation of the comparative results of phospholipid turnover in normal epidermis and a transplantable squamous cell carcinoma, Costello *et al.*(5) were not able to show a difference in specific activity of the 2 tissues *in vivo* using P^{32}. However, total activity was shown to be higher in the tumor due to an elevated phospholipid phosphorus content. Since our data were obtained using tumor tissue that was induced by painting with 20-methylcholanthrene the data from the 2 studies are not strictly comparable. Yet the turnover rate of phosphorus in phosphatido-peptide was much greater in tumor tissue than the corresponding normal tissue. We believe the effects reported are due to growing tumor since no effect was observed in mice painted with 20-methylcholanthrene until after the appearance of papillomas. In addition, there was no effect due to topical treatment with benzene, the solvent for 20-methylcholanthrene. These data indicated that a metabolic characteristic of neoplastic tissue can be demonstrated in histologically normal epidermis from animals with squamous cell carcinoma. It is of interest that Clark *et al.* (6) reported recently that premalignant and malignant lesions of the human vulva preferentially take up P^{32}.

Summary. Evidence was presented which demonstrates that the turnover of phosphorus in phosphatido-peptide is greater for neoplastic epidermis than normal epidermis. Histologically "normal" epidermis obtained from mice bearing a squamous cell carcinoma induced with 20-methylcholanthrene had a

phosphorus turnover more closely resembling neoplastic than normal tissue. Further studies concerning the biochemical changes mentioned are in progress.

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Effect of Bile Acids on Serum Cholesterol in the Chick.*† (26741)

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Recently Beher *et al.*(1) and Howe *et al.* (2) have reported that several bile acids (hyodeoxycholic acid, chenodeoxycholic acid and lithocholic acid) counteract the hypercholesterolemic effect of cholic acid in mice and prevent cholesterol accumulation in the liver and carcass. Portman and Bruno(3) have reported similar findings with rats. Experimental work on the effect of cholic acid or any other bile acids on serum cholesterol levels in chickens has not been carried out. Since the chicken is known to secrete quite different bile acids than the mouse and rat, it was desirable to investigate the effect of feeding various bile acids on serum cholesterol in chickens.

Experimental. The experiment was conducted with day-old cross-bred cockerel chicks (Cornish male X White Plymouth Rock female) obtained from a local hatchery. They were housed in electrically heated batteries with wire floors and were supplied feed and water *ad libitum*. They received the experimental diets for 3 weeks and were weighed at weekly intervals during this period. At termination of the growth phase of the experiment, blood samples were obtained by heart puncture and the serum separated for total cholesterol determination by the method of Schoenheimer and Sperry(4). The cholic acid, lithocholic acid and hyodeoxycholic acid used in the experiment were obtained from Nutritional Biochemicals Corp. These bile acids were added to the diet at the 0.2% level singularly and combinations of cholic and lithocholic acids, and cholic and hyodeoxycholic acids. The basal diet used was similar to that used in earlier work(4), modified to contain 4.0% corn oil. All substitutions in the diet were made at the expense of fiber.

Three groups of 8 chicks each were started on each of the dietary treatments. The results were analyzed for statistical differences by the Multiple Range Test(5).

Results. The effect of feeding the various bile acids on growth and serum cholesterol is shown in Table I. Addition of lithocholic acid to the basal diet caused a marked depression in growth rate. When cholic acid was added to the diet containing lithocholic acid this retardation in growth rate was reversed. Addition of cholic acid, hyodeoxycholic acid or a combination of these 2 bile acids to the basal diet had no statistically significant effect on growth rate of cockerels. However, it should be noted that rate of growth was faster when both hyodeoxycholic acid and the combination of hyodeoxycholic acid and cholic acid were added to the basal diet.

While lithocholic acid depressed growth, it caused an approximately 3-fold increase in serum cholesterol level of the cockerels. Addition of cholic acid to the diet containing

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TABLE I. Effect of Bile Acids on Growth and Serum Cholesterol of Chicks.

Supplements to basal diet	Avg wt 3 wk, g	Cholesterol/100 ml of serum, mg
None	307 ± 7	178 ± 7
.2% cholic acid	298 ± 7	181 ± 7
" lithocholic acid	176 ± 8	575 ± 26
" hyodeoxycholic acid	319 ± 9	189 ± 9
" cholic acid + 2% lithocholic acid	294 ± 11	298 ± 15
" cholic acid + 2% hyodeoxycholic acid	330 ± 9	169 ± 7

Grouping of treatment results*

Body wt	176	294	298	307	319	330
Serum chol.	169	178	181	189	298	575

* Two values not underscored by the same line are significantly different ($P < .05$).

lithocholic acid appears to have caused a reduction in this hypercholesteremic condition. However, the serum cholesterol level was still approximately twice the normal value. Addition of cholic acid, hyodeoxycholic acid or a combination of the two did not affect serum cholesterol significantly as measured by the statistics applied. A small, non-significant drop in serum cholesterol was observed in chicks receiving the combination of cholic acid and hyodeoxycholic acid.

Discussion. The results indicate in light of other studies that striking species differences may be expected with respect to the effect on serum cholesterol when various bile acids are added to the diet. Howe *et al.*(2) found that lithocholic acid was very effective in lowering serum cholesterol in mice, while the results reported here with chickens are the reverse. It seems possible that this may be caused by differences in the type of bile acids excreted by the 2 species. Haslewood and Wootton(6) reported finding only cholic acid in the mouse (*Mus musculus*), while Japanese workers (reviewed by Anderson, Haslewood and Wootton(7)) have found a large array of bile acids in the domestic chicken. They are chenodeoxycholic acid, isolithocholic acid, tetrahydroxynorsterocholanic acid, 3 α-hydroxy-7-oxocholanic acid and 3-oxochola-4:6-dienic acid. No investigations have been conducted with the chick on production of bile acid artifacts by circulation of the original "native" bile salts through the enterohepatic circulation, or by intestinal micro-organisms. Therefore, it cannot be known exactly which of the above bile acids are "natural" to the domestic chicken. If regulation of bile acid production in the domestic fowl is a homeostatic process as indicated for the rat(8), it may be that lithocholic acid interferes with absorption of chenodeoxycholic acid or some other bile acid that plays a part in the homeostatic control in chicks, or the lithocholic acid itself may be absorbed and play a role in the homeostatic control in the chicken.

Portman and Bruno(9) have reported on the effects of feeding a number of bile salts to rats. They found that all the bile acids tested that had serum cholesterol-elevating activity contained a 12α-hydroxy grouping. Their results are in close agreement with those of Howe *et al.*(2) with respect to activity of various derivatives of cholic acid to raise the serum cholesterol of mice. It is obvious from the results presented in this paper that the theory that bile salts must have a 12α-hydroxy grouping to have serum cholesterol-elevating activity will not apply with the domestic chicken.

Summary. Addition of cholic acid to the diet of chicks does not cause a rise in serum cholesterol as has been reported in mice and rats. Addition of lithocholic acid to the chicken diets caused a marked increase in serum cholesterol. This effect is opposite to that reported to occur in mice and rats.

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A Rapid, Reproducible and Sensitive Levator Ani Test for Anabolic Activity.* (26742)

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It was recently demonstrated that an anabolic steroid could, within 39 hours after injection, induce a 4-fold increase in uptake of a labelled non-metabolizable amino acid by the levator ani muscle of the rat(1). The possibility of using this phenomenon as a rapid indicator of the anabolic potential of various steroids was therefore explored. The effect of 8 different steroids on uptake of the amino acid, alpha amino-isobutyric acid-1-C^{14} (AIB), was determined. The data indicate the new procedure to be more rapid than the standard levator ani test; it is also more sensitive and highly reproducible. Several other features of interest were noted, including the diminished excretion of AIB. It is suggested that excretion of AIB might also serve as an indicator of anabolic activity.

Method. Male rats of the Holtzman strain weighing approximately 150 g were kept in the laboratory on a diet of Rockland pellets until their weight was 200 ± 10 g. In each series of experiments the rats were divided into groups of 6 approximately equal in total weight. One group was given diluent only and served as the control, the second group was given testosterone propionate, 50 mg/kg, and served as the standard, and the other experimental group or groups were given the test steroids in the same dosage. Immediately after castration under ether anaesthesia the steroids or diluent were given intramuscularly. The animals were then subdivided into groups of 3 and placed in metabolic cages for collection of the pooled urine. Food was withheld but water was available. Thirty hours after castration each animal was given subcutaneously 1.0 ml of the AIB solution containing 550,000 ± 5,000 counts (specific activity 0.1 mC/mM). Nine hours later the animals were exsanguinated under ether anesthesia by direct cardiac puncture. The levator ani were excised, weighed, and individually homogenized with 3.0 ml of acetic acid (pH 3.5). The other organs and tissues were also excised, weighed and homogenized, in pools of 3 corresponding to the metabolism cage pools, with a proportional volume of acetic acid. After centrifugation the supernatants were plated in 0.5 ml aliquots and counted to a total of 1000 counts 3 times in a thin window gas flow counter with sample changer and printing timer.† One hundred ml of hot water was used to wash down the metabolism cages, added to the corresponding pooled urine, made up to a total volume of 200 ml and a 0.5 ml aliquot counted. Total nitrogen in the urine was determined by the microKjeldahl method. The individual blood serums were precipitated with tungstic acid and a 0.5 ml aliquot of the filtrate counted.‡

Results. The results of the 4 series of experiments are given in Tables I and II. All of the animals lost 15% in body weight as a result of castration and food deprivation. Nevertheless, all of the levator ani responded to the stimulation of the steroids in the 39 hours of experiment by an increase in weight and by an increase in uptake of AIB (Table I). Control levator ani weights ranged from an average of 63 to 87 mg and experimental ones from 110 to 134 mg. Ratios of experimental to control weights ranged from 1.35 to 2.13 and net increase in weight from 35 to 113% with an average of 50%. The uptake of AIB by control levators was 1,780 to

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† The sample changer and printing timer were purchased with a grant from the Ciba Co. through the courtesy of Dr. C. H. Sullivan.

‡ This filtrate was chosen because it gave a count recovery of 93%; serum plated directly gave a recovery of only 32% and often caused difficulty because of flaking and curling; trichloracetic acid filtrate was deliquescent when plated and recovery with it only 41%.

TABLE I. Levator Data.

Series	Steroid*	No. of rats	Avg body wt, g		Levator ani wt				Levator ani uptake			
			Original	Terminal	Avg mg	S.E., % of mean	Ratio: To control	Ratio: To test. prop.	Avg counts per g	S.E., % of mean	Ratio: To control	Ratio: To test. prop.
I	Methandrostenolone	6	195	168	108	4.7	1.72	.81	4,730	3.6	2.43	.62
	Testosterone propionate	6	196	165	134	2.2	2.13	—	7,590	2.8	3.89	—
	Control	6	200	163	63	12.4	—	.47	1,950	5.1	—	.26
II	Norethandrolone	6	211	178	125	8.9	1.44	1.01	5,750	4.8	3.23	.81
	Norethandrolone propionate	6	203	173	117	6.1	1.35	.95	6,660	4.4	3.74	.93
	Testosterone propionate	6	204	173	124	6.7	1.43	—	7,150	7.7	4.01	—
	Control	6	208	182	87	11.5	—	.70	1,780	3.6	—	.25
III	Nandrolone phenpropionate	6	198	171	115	5.4	1.44	.96	7,100	4.4	3.88	1.01
	Norpropandrolate	6	205	174	113	6.1	1.40	.94	7,110	5.9	3.88	1.01
	Testosterone propionate	6	205	171	120	5.1	1.48	—	7,040	4.7	3.86	—
	Control	6	197	171	81	5.3	—	.68	1,830	5.0	—	.26
IV	Testosterone	6	193	164	110	5.5	1.35	.95	8,270	4.0	4.35	1.08
	Testosterone enanthate	6	193	162	112	5.2	1.38	.97	7,630	3.9	4.02	.98
	Testosterone propionate	6	197	167	116	5.5	1.43	—	7,780	4.8	4.09	—
	Control	6	192	164	81	4.2	—	.70	1,900	5.0	—	.24

* Methandrostenolone: 17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one;
Kindly supplied by Dr. C. H. Sullivan, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Norethandrolone: 17α-ethyl-17β-hydroxy-19-norandrost-4-en-3-one;
Norethandrolone propionate: 17α-ethyl-4-estrene-3β,17-β-diol-3-propionate;
Norpropandrolate: 4-estrene-3β,17β-diol-3,17-dipropionate;
Kindly supplied by Drs. I. C. Winter, R. L. Craig, and T. H. Hayes, G. D. Searle and Co., Chicago, Ill.

Nandrolone phenpropionate: 19-nor-$\triangle^4$-androstene-17β-ol-3-one-β-phenyl propionate;
Kindly supplied by Dr. H. A. Strade, Organon, Inc., Orange, N. J.

Testosterone enanthate: Testosterone-17-β-n-enanthate;
Kindly supplied by Mr. E. Grossman, E. R. Squibb and Sons, New York, N. Y.

Testosterone propionate and testosterone: Stock material from the hospital pharmacy; the latter compound was suspended in water, all other compounds were dissolved in oil.

TABLE II. Urine Data.

Series	Steroid	No. of rats	Urine nitrogen Avg mg/rat	Urine nitrogen Ratio To control	Urine nitrogen Ratio To test. prop.	Urine AIB Avg counts/rat	Urine AIB Ratio To control	Urine AIB Ratio To test. prop.
I	Methandrostenolone	6	227	1.23	1.08	3,500	.83	1.40
	Testosterone propionate	6	211	1.14	—	2,500	.60	—
	Control	6	185	—	.88	4,200	—	1.68
II	Norethandrolone	6	202	.78	1.01	3,700	.48	1.39
	Norethandrolone propionate	6	198	.76	.99	3,200	.42	1.20
	Testosterone propionate	6	200	.77	—	2,670	.35	—
	Control	6	259	—	1.30	7,670	—	2.87
III	Nandrolone phenpropionate	6	202	1.09	1.01	2,070	.36	.78
	Norpropandrolate	6	220	1.19	1.10	2,800	.49	1.05
	Testosterone propionate	6	200	1.08	—	2,670	.47	—
	Control	6	185	—	.93	5,700	—	2.13
IV	Testosterone	6	200	1.01	1.09	6,700	.47	1.33
	Testosterone enanthate	6	214	1.08	1.16	7,920	.56	1.58
	Testosterone propionate	6	184	.93	—	5,030	.36	—
	Control	6	198	—	1.08	14,140	—	2.81

1,950 counts per gram and 4,730 to 8,270 for experimental groups. Ratios of experimental to corresponding control uptakes were 2.43 to 4.35. Net increases in uptake varied from 143 to 335% with a mean of 276%. Thus, average percent increase in uptake was 5½ times as great as average percent increase in weight.

Urinary excretion data are given in Table II. In the 39 hours of experiment nitrogen retention was manifest only in series II. The other series showed no change or an actual increased loss over controls. The average for the ratios of experimental to control nitrogen excretion for the 4 series was 1.01. Urinary excretion of the labelled compound was, however, decreased in all of the experiments in the 9 hours following its administration. Ratios of experimental to control excretions ranged from 0.83 to 0.35 or a decrease of 17 to 65% with an average of 51%. Thus, although there was no consistent decrease in nitrogen excretion as a result of administration of the steroids, there was a significant reduction in excretion of the AIB.

The changes in uptake of the other tissues analyzed for AIB were small in comparison to the levators. Serum, diaphragm, kidney and heart showed variable small changes, the experimental to control ratios being 1.03, 0.98, 0.95, and 1.06 respectively. Skeletal muscle (tongue was used) showed a consistent increase and liver and thymus a consistent decrease. The respective experimental to control ratios were 1.10, 0.88 and 0.86. These represent a 10 to 14% change which is only one-twentieth of that of the levator muscle.

The reproducibility of the method within the given groups is indicated by the standard errors in Table I. These are given in percent of the corresponding means to allow comparison between the two factors, weight gain and AIB uptake. The mean of the standard errors for the 15 groups is 6.3 (S.E. $\pm$ 0.73) for the levator weights and 4.6 (S.E. $\pm$ 0.29) for the uptakes. The latter is thus somewhat more accurate than the former. The reproducibility of the uptakes in the replicated groups (control and testosterone propionate) is indicated by the narrow range of the actual count values and the ratio values. Average counts in the control levator groups for the 4 series were 1,950, 1,780, 1,830 and 1,900 and for the 4 testosterone propionate groups, 7,590, 7,150, 7,040, and 7,780. The testosterone propionate to control ratios were 3.89, 4.01, 3.86 and 4.09 and the reciprocal ratios were 0.26, 0.25, 0.26 and 0.24 respectively.

Discussion. On the basis of the above results the levator ani-AIB test would appear to be as effective as the standard levator ani weight tests for determining the anabolic po-

tential of steroids. It has, in addition, a number of advantages over these tests, the Eisenberg-Gordan test and the modified test of Hershberger(2,3). The former requires 28 days for its performance and the latter 7 days, whereas the AIB test requires only 39 hours. The 30-hour interval from steroid injection to AIB administration was adopted to be sure of maximum effectiveness of the steroid, and the 9-hour interval from AIB administration to sacrifice to ensure an adequate circulating level of this compound; its previously determined half time in the circulation was 9 hours. Since anabolic steroids probably become effective soon after injection it may be possible to shorten the AIB test time. Studies are now being carried out to determine the shortest time after steroid administration in which statistically valid results are obtainable with the test. Admittedly, the test requires a little more working time (to process the levators for counting) than the simpler weighing test but it obviates the need for housing and feeding groups of rats for long periods of time and the need for daily injections of the test steroids. Another advantage of the AIB test, perhaps minor, is that precise excision of the levator ani is not critical to the test—the effect being measured not in total counts but in counts per gram of the muscle.

In this series of experiments the AIB test is somewhat more accurate than the weight test. The difference noted above between the means of the standard errors of the counts and those of the weights in the 15 experiments is statistically significant ($p < 0.05$). The reproducibility of the counts (per gram) in the 4 replicated control and testosterone propionate experiments is quite good. The coefficient of variation between the 4 values in the former was 4.7%, in the latter 2.7%, and between the respective ratios, 3.8%. The sensitivity of the AIB test is 5½ times that of the weight test on the basis of the ratio between percent increase in uptake to percent increase in weight. This sensitivity may actually be somewhat higher. If the first 2 weight ratios and the first uptake ratio are excluded from their respective columns (this is valid on statistical grounds) average net uptake increase would be 290% and average net weight increase would be 41% with a ratio of seven. Thus, AIB uptake is 5 to 7 times as sensitive as weight increase as an indicator of anabolic potential in the 39 hours of these experiments.

There was an appreciable reduction in urinary excretion of the AIB in the 9 hours after its administration but no consistent change in nitrogen excretion in the 39 hours after steroid administration. The explanation for this reduction in urinary AIB is not clear from these experiments. Although the relative kidney counts were 5 times as high as the average for the other soft tissues analyzed (20,000 *vs* 4,000 counts/g) the experimental animal kidney counts were the same as the controls. Also, the 10% increase in uptake by the skeletal muscle was not reflected by any change in the blood level which could have affected the excretion. This considerable reduction in AIB excretion, however, suggests the possibility of using this change as an added or alternate indicator of the activity of anabolic steroids. Prolonging the period of the test until nitrogen retention becomes established would allow quantitative correlation between AIB excretion and nitrogen retention to be determined and AIB itself could then serve as a monitor for nitrogen sparing.

At the high steroid dose level used there was less than a 2-fold spread, 2.43 to 4.35, in uptake ratios. Taking the mean, 3.96, of the 4 testosterone propionate ratios as only one value the mean of the ratios for the 8 compounds tested was 3.76 with an S.D. of ± 0.56 (15%). The 2 non-esterified compounds, methandrostenolone and norethandrolone, with known low androgenic potency had ratios below this mean (2.43, 3.23) and the parent compound, testosterone, with known high androgenic and anabolic activity had a ratio above this mean (4.35). The ratios of the 5 esterified compounds, on the other hand, were grouped very closely (3.96, 3.74, 3.88, 3.88, 4.02) with a mean of 3.90 and an S.D. of ± 0.13 (3.4%). It is interesting that the 2 nortestosterone esters, the phenpropionate and the dipropionate had identical values (3.88, 3.88), that the 17-

ethyl nortestosterone ester had a value very close to these (3.74), and that the 2 testosterone esters had practically identical values to each other (3.96, 4.02) and to the 3 nortestosterone values. Thus, deletion of the methyl group, presence of the 17-ethyl group, esterification at the 17 position, esterification at both the 3 and 17 positions and the nature of the esterifying groups did not seem to affect the common action of these compounds which is being expressed as an increase in uptake of the labelled amino acid. What proportion of uptake of the AIB is attributable to the anabolic effect and what proportion to the androgenic effect of these steroids cannot be deduced from these data. Nevertheless, the degree of agreement among the 5 esterified compounds suggests that the various compounds have the same effect on the uptake or concentrating mechanism or that their metabolic breakdown results in a common compound causing the effect noted.

Summary. 1. A test for determination of the anabolic potential of steroids using increase in uptake of a C^{14} labelled non-metabolizable amino acid by the levator ani muscle of the rat is described. 2. This test is considerably more rapid than the 2 standard levator ani weight increment tests. Reproducibility of the method within a given test is quite good as is reproducibility in serial tests with a given steroid. The sensitivity of this test is 5- to 7-fold that of the weight increment test. 3. The reduction in urinary AIB excretion observed even before any nitrogen retention becomes established suggests the possibility of using this reduction as an aditional or alternate indicator of the anabolic activity of steroids. 4. The close agreement in uptake ratios with 5 different steroid esters suggests that the metabolic product ultimately effective in causing the increase in uptake of the labelled compound is the same or that the various steroids have the same effect on the uptake mechanism.

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Effect of Size and Concentration of Latex Particles on Respiration of Human Blood Leucocytes.* (26743)

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Recent workers on physical and biochemical aspects of phagocytosis have used suspensions of polystyrene latex particles as the test substance(1,2). Using guinea pig exudate leucocytes and polystyrene latex particles 1.171 μ in diameter, Sbarra and Karnovsky concluded that phagocytosis requires energy, and that the oxygen consumed by the test cells is directly proportional to the number of particles ingested by them(2). Strauss and Stetson have noted the effect of 0.802 μ polystyrene latex particles and solutions of endotoxins of Gram negative bacteria on the respiratory activity of leucocytes of human peripheral blood(1).

The present paper describes effect of size and concentration of polystyrene latex particles upon oxygen consumption of leucocytes of heparinized human peripheral blood.

Materials and methods. Blood. Normal human peripheral blood was drawn and heparinized as described elsewhere(1). Blood

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TABLE I. Physical Properties of Suspensions of Polystyrene Latex Particles.

	Dow Lot #	Size of particle (μ)	Vol of particle (ml)	% solids by wt	s.g. of suspension	Concentration particles/ml
1	LS–040A	.088	3.61×10^{-16}	9.42	.982	2.66×10^{14}
2	LS–055A	.188	3.48×10^{-15}	10.30	.966	3.06×10^{13}
3	LS–057A	.264	9.63×10^{-15}	10.20	.964	1.10×10^{13}
4	LS–061A	.365	2.55×10^{-14}	9.99	.962	4.07×10^{12}
5	LS–063A	.557	9.05×10^{-14}	12.91	.964	1.48×10^{12}
6	LS–066A	.814	2.84×10^{-13}	11.20	.984	4.00×10^{11}
7	LS–067A	1.171	8.43×10^{-13}	10.26	.956	1.27×10^{11}

from one donor was used for all experiments in the interest of consistent quantitative responses. However, various donors responded similarly in a qualitative sense. *Polystyrene latex particles.* Samples of 7 graded sizes of particles were obtained from Dow Chemical Co., Midland, Mich., through the courtesy of Dr. John W. Vanderhoff. Each sterile sample contained particles of uniform diameter suspended in a buffered saline solution, and was stored at 4°C. From data supplied by Dow Chemical Co., the concentration of each stock suspension was calculated§ (Table I). However, investigators should be aware that suspensions must not be used after storage over many months, for there will be fewer particles present than originally, owing to destructive action by bacteria which have gained entry when vials are opened to withdraw samples. Different concentrations of latex suspensions were prepared by dilution with physiologic saline. *Manometric determinations.* Oxygen consumption was measured by the direct method of Warburg, and as in previous experiments(1) 2 ml whole heparinized blood was used per flask. 0.2 ml latex suspension was pipetted into the sidearm of triplicate sets of flasks. After a 90 minute equilibration period, the suspensions were added. The value at 30 minutes after addition is used for interpretation because recent investigations(1,3,4) suggest that the significant portion of the phagocytic event occurs within this time period.

Results. Series A: Different sized particles were studied in a final concentration of 6.35 $\times 10^{10}$ particles/ml. Control values for blood without latex were constant at 7 μl $\pm$ 0.5 μl oxygen uptake per 2 ml of blood at 30 minutes. Stimulation of oxygen uptake was directly proportional to the size of the polystyrene latex particle within the range 0.264 μ to 1.171 μ. Particles smaller than 0.264 μ produced no significant increment in leucocyte respiration (Table II).

Series B: Experiments were designed to determine the relationship between concentration and oxygen uptake for 3 different sizes of particles and also to confirm observations of series A (Table III). When size was held constant, increase in oxygen uptake was directly proportional to the concentration of the three sizes of particles tested. On the other hand when concentration of particles was held constant, larger particles result in a greater increase in oxygen uptake over control values, as in series A.

Further experiments using particles 0.088 μ, 0.188 μ and 0.264 μ have shown no stimulation of respiration by the first 2 sizes, and erratic stimulation of respiration by the last size, even when undiluted stock suspensions were tested.

Discussion. Polystyrene latex particles

TABLE II. Effect of Size of Particles upon Leucocyte Respiration. Concentration constant at 6.35 $\times 10^{10}$ particles/ml.

Group	No. of determinations	Size of particle (μ)	μl increase in O_2 uptake/2 ml blood over control values at 30 min.
1	2	.088	.5 $\pm$ .7
2	2	.188	.5 $\pm$ .7
3	2	.264	3.3 $\pm$ 1.1
4	3	.365	7.4 $\pm$ 2.4
5	3	.557	12.3 $\pm$ 1.3
6	3	.814	38.8 $\pm$ 1.4
7	2	1.171	46.6 $\pm$ 3.5

§ $$\frac{\text{No. particles}}{\text{ml}} = \frac{\text{g solids}}{\text{ml suspension}} \times \frac{1}{\text{s.g. suspension}} \times \frac{1}{\text{vol each particle}}$$

TABLE III. Effect of Concentration of Particles upon Leucocyte Respiration for 3 Sizes of Particles.*

Group	Size of particle (μ)	μl increase in O_2 uptake over control values at 30 min. Concentration particles/ml, 12.7×10^{10}	Concentration particles/ml, 6.35×10^{10}	Concentration particles/ml, 1.05×10^{10}
1	.365	13.3 ± 1.1	7.4 ± 1.7	1.5 ± 1.9
2	.814	45.7 ± 3.7	37.5 ± 4.7	8.8 ± 2.3
3	1.171	47.7 ± 2.3	48.0 ± 5.0	13.9 ± 1.4

* Each oxygen uptake value represents mean of 3 experiments.

possess physical properties which lend themselves to investigation of the mechanics of phagocytosis. They have been characterized as inert(2). They are available in rigidly standardized suspensions containing particles highly uniform in diameter. With the exception of the 3 smallest sizes they are easily visible with the phase microscope.

Preliminary phase microscopy observations have not resolved the problem of how many latex particles enter the leucocytes. It is extremely difficult to count the number of particles within cells owing to the presence of optically similar cytoplasmic constituents. While Table III shows a direct proportionality between particle concentration and respiration for particles of 3 selected sizes, the failure of the 2 smallest sizes of particles to be associated with significant increases in oxygen uptake by leucocytes probably indicates that little or no phagocytosis of these particles occurred. Thus a critical particle size is defined in this system; below it, addition of particles to whole blood regardless of particle concentration fails to stimulate leucocyte respiration. This may explain earlier results in which heat denatured bovine albumin failed to stimulate leucocyte respiration (1).

Latex particles have proved useful in quantitating some mechanical aspects of phagocytosis. The information gained in regard to size and concentration now lends itself to the study of factors involved in the phagocytic event which are not strictly mechanical in nature. The system described permits further studies in phagocytosis in which the role of certain opsonic factors may be assessed; latex particles coated with various globulin fractions are being used at present to delineate this role.

Summary. Rigorously standardized polystyrene latex particles of graded sizes were added to whole blood, and oxygen uptake by leucocytes as they ingested these particles was measured by the direct method of Warburg. The importance of both size and concentration of particles was established.

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Humoral Regulation of Erythropoiesis VI. Mechanism of Action of Erythropoietine in the Irradiated Animal. (26744)

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We previously reported an assay for erythropoietine using the sublethally irradiated animal(1). The results with this assay differ from those using fasted(2) or transfused animals(3); in the irradiated animal there is a longer interval between injection of erythropoietine and a measurable effect. The reason for this difference is unexplained. It has been suggested that erythropoietine promotes differentiation of stem cells(4,5), stimulates division in the erythroid compartment(6), or both(7). In this paper further studies on the response of the irradiated animal to erythropoietine are reported. These data support the concept that erythropoietine promotes stem cell differentiation; a tentative hypothesis is proposed to explain the difference in rate of response of irradiated and transfused animals.

Material and methods. Female Sprague-Dawley rats weighing 135-160 g were used. Irradiation was given with a 3.0 MEV Van de Graaff linear accelerator at a dose rate of 300 r/minute as measured by a Bendix ionization chamber known to be air equivalent at this energy. Transfused animals were given red cells in an amount equivalent to 1.4% of body weight 4 days prior to administration of erythropoietine. Erythropoietine was obtained from the urine of patients with congenital hypoplastic anemia and concentrated by evaporation or precipitated with 80% alcohol; the concentrates were dialyzed against normal saline at 4°C prior to use.

Red cell production was estimated by reticulocyte counts and Fe^{59} incorporation curves. In the Fe^{59} incorporation studies, we injected intramuscularly 5 mg of non-radioactive iron, as Imferon, 3 hours after injection of Fe^{59}. The dose of Imferon was repeated daily thereafter. By diluting out the Fe^{59} the non-radioactive iron minimizes the continued utilization of radioiron recycled through the tissues. Blood volume was measured in each animal by injecting Cr^{51} labelled cells 20 minutes prior to exsanguination; Cr^{51} and Fe^{59} counts were separated using a single channel spectrometer.

Results. 1. *Effect of erythropoietine after 200 r.* Twenty-four hours after 200 r whole body radiation total Fe^{59} incorporation is at a minimum. When Fe^{59} is given 48 hours after irradiation few labeled cells emerge during the following 24 hours but a substantial number emerge between 24 and 48 hours (72-96 hours post-radiation). Administration of 2-4 CS units of erythropoietine(8) immediately after irradiation and 24 hours thereafter affected the cohort of cells emerging between 24 and 48 hours after Fe^{59} (72-96 hours post-radiation) but there was no detectable effect within the first 24 hours after Fe^{59} (72 hours post-radiation) (Table I). A difference in reticulocytes was not detectable until 96 hours after initial injection of erythropoietine (Table II).

2. *Effect of erythropoietine after 400 r.* Erythropoiesis does not resume until several days after 400 r; reticulocyte count rises substantially on the 6th day. Administration of a single dose of erythropoietine immediately after 400 r produces an earlier recovery (Table III). A single dose of erythropoietine given 24 hours after irradiation was no more effective than the dose given immediately after irradiation (Table III). The iron in-

TABLE I. Effect of Erythropoietine (EP) on Recovery of Erythropoiesis in Rats Receiving 200 r.

Treatment	Hr after Fe^{59} 24	48	72
Control	5.2 ± .73*	13.5 ± 1.83	20.2 ± 1
EP × 2†	6.8 ± .83	20.4 ± .52	26.4 ± .51
EP daily	6.8 ± .83	21.5 ± .53	26.9 ± .61

* Iron incorporation as % of inj. dose. Values are mean of 18 animals ± S.E. Fe^{59} given 48 hr after irradiation.

† Erythropoietine given immediately and 24 hr after irradiation.

TABLE II. Effect of Erythropoietine on Reticulocytes after Sublethal Radiation.

Treatment	Hr after 200 r 72	96	120
Control	.3 ± .06†	.8 ± .13	2.0 ± .25
EP × 2*	.3 ± .04	1.3 ± .18	2.7 ± .32
EP daily	.2 ± .05	1.2 ± .13	3.7 ± .36

* Erythropoietine given immediately and 24 hr after irradiation.

† Reticulocyte percentage ± S.E. Each value based on 12 animals.

corporation curves in 4 experiments showed similar changes (Table IV).

3. *Effect of erythropoietine in the transfused rat.* A single dose of erythropoietine produced a small increase in reticulocytes within 48 hours with a very pronounced effect by 72 hours (Table V). Multiple injections produced an even greater effect. Fe^{59} data were consistent with reticulocyte values.

TABLE III. Effect of Erythropoietine after 400 r.

Treatment	Reticulocytes Day post-radiation 6	7	8
EP immediately after irrad.	1.94 ±.3*	4.67 ±.8	6.79 ±1.23
Control irradiated	.31 ±.09	1.9 ±.34	5.01 ± .66
EP 24 hr after irrad.	.6 ±.19	3.45 ±.91	6.61 ± .45

* Mean of reticulocytes of 6 animals ± S.E.

Discussion. Previously we proposed a model for erythropoiesis(9) in which the erythroid compartment is replenished from a primitive or stem cell compartment. After differentiation into erythroid elements, maturation and division are asynchronous. Some cells and their progeny will divide more than others; some may mature without dividing. Cell death may occur during maturation and under some circumstances may be substantial. The sequence of events in the erythroid compartment following hypertransfusion suggests that the suppression results primarily from a decreased input from the stem cell compartment(10). Irradiation affects erythropoiesis by destruction of cells within the erythroid compartment as well as in the stem cell compartment.

TABLE IV. Effect of Erythropoietine on Recovery of Erythropoiesis in Rats Receiving 400 r.

	Hr after Fe^{59} 24	48	72
Control	8 ± 1.21†	18 ± 2.13	20 ± 1.93
EP*	15 ± 1.73	30 ± 1.86	26 ± 2.05

* Erythropoietine given immediately after irradiation; Fe^{59} labeled plasma given 5 days after irradiation.

† Percentage of inj. iron incorporated in red cells; mean of 24 animals ± S.E.

TABLE V. Reticulocyte Response of Hypertransfused Rats to a Single Injection of Erythropoietine.

Hr after treatment	Erythropoietine	Control
24	.13 ± .04*	.21 ± .04
48	.40 ± .09	.25 ± .05
72	1.78 ± .32	.15 ± .03
96	3.10 ± .7	.17 ± .06
120	1.91 ± .11	.14 ± .02

* Avg reticulocyte percentage in 5 animals ± S.E. of mean.

The response of the hypertransfused animals to erythropoietine seems best explained by the differentiation of large numbers of stem cells. The emergence of some cells as early as 24-48 hours after a strong stimulation suggests either a shortening of generation times or skipped divisions (*i.e.* maturation without division). Alpen(4) has presented evidence to suggest that even after severe stimulation the generation time of the erythroblast is unchanged. Whether this applies to later stages remains moot. A primary effect on later stages of development is unlikely since there are few such cells remaining and certainly not enough to explain the magnitude of the response unless the generation times are incredibly short. Jacobson's studies in the mouse(3) where erythroid elements are reported to be completely absent after hypertransfusion lend further support to the notion that erythropoietine promotes stem cell differentiation.

The data clearly demonstrate a difference between the effect of erythropoietine in irradiated and hypertransfused animals. The effect was delayed after 200 r but much more so after 400 r. It seems unlikely that this could result from continued availability of

erythropoietine for several days during which time the stem cell compartment regenerated. Estimates of a circulating half time for erythropoietine of 5-10 hours in irradiated animals seem incompatible with such a thesis(11). Also, these estimates represent a maximum since they were derived from studies in which there was undoubtedly an overlap between production and destruction of erythropoietine. Furthermore, administration of erythropoietine 24 hours after irradiation was no more effective than when given immediately after irradiation, contrary to what would be expected if there were continued availability.

A more attractive hypothesis is that erythropoietine when given immediately after irradiation differentiates a portion of the stem cell compartment, which after one or 2 abortive attempts at division dies. The resultant depopulation of the stem cell compartment serves as a stimulus for earlier regeneration of the stem cell compartment(12). This explanation entails certain assumptions, which, while unproved, are compatible with current knowledge. The erythroid compartment is not self-sustaining but is constantly replenished from a stem cell pool, which turns over at a slower rate than the erythroid compartment. Rate of turnover of the stem cell compartment is determined by a feedback mechanism governed by rate of removal of stem cells; cells may be removed by death within the compartment or by differentiation. After irradiation cells in both the erythroid and stem cell compartment are lost either directly or upon entering mitosis. The damage to the erythroid compartment is greater than to the stem cell compartment due to differences in sensitivity perhaps reflecting differences in generative cycle. Following radiation injury the number of cells killed directly, dying during abortive division, and surviving is dependent on dose. It is further suggested that at higher doses some surviving cells for a period are viable but temporarily "infertile," and others permanently sterile, the proportion being dose dependent. These cells at least temporarily retain the capacity to differentiate. The stage of "repair" involves restoration of the capacity of division of those cells "temporarily infertile." Alternatively, the latter might be considered as "undamaged"(12) but this seems unlikely at high doses. The period for repair is also dose dependent. Once the capacity for division has been renewed, rate of division and regeneration will again be governed by the feedback mechanism and be dependent on the numbers of cells in the compartment, *i.e.*, surviving but sterile cells would influence rate of regeneration. Erythropoietine by reducing the number of the latter cells would permit a more rapid regeneration.

Summary. Erythropoietine produces an increase in reticulocytes in transfused animals within 48-72 hours. In irradiated animals the response is delayed, length of delay being in part dose dependent. A change in reticulocytes is not observed until 96 hours when erythropoietine is given immediately after 200 r; after 400 r the effect is first noted at 6 days. A tentative hypothesis is proposed to explain these results. It is suggested that erythropoietine promotes differentiation of stem cells into erythroid elements and that depopulation of the stem cell compartment stimulates division within that compartment.

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Binding of Radioactive Sodium, Potassium, and Bromide in Guinea Pig Brain Homogenates.* (26745)

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Binding of various electrolytes in the brain has been described over the years since Fremy in 1841 first noted the presence of fixed mineral substances in brain tissue. Thudichum found potassium to be the chief inorganic constituent present in protagon and demonstrated that calcium is attached to cephalin. Koch and Pike(7) showed that cephalin contains more potassium than sodium, but that in lecithin the reverse is true.

Christensen and Hastings(3) found that cephalin has the ability to combine with sodium and potassium with equal affinity in amounts increasing with pH. At neutrality they found that 0.5-0.6 equivalents of alkali were bound per mole of cephalin.

Folch(4) reported 30% excess of cations over anions in brain, accounting for this "anion deficit" by the binding of approximately this proportion of brain cations to cerebron sulfuric acid and phosphatidyl serine. Twenty per cent of the brain sodium (10 mEq) was bound to phosphatidyl serine.

Stone and Shapiro(13) filtered homogenates of brain and muscle under negative pressure at 3-4°C and found that 20% of brain and muscle potassium did not diffuse through cellophane, while 100% of sodium was freely diffusible. Bergen, Stone and Hoagland(2) used the ultrafiltration technic of Stone and Shapiro to demonstrate binding of about 30% of rat brain potassium not influenced by adrenalectomy. Their results confirmed earlier reports of a slowly exchanging brain potassium compartment which develops with age in rats. On the other hand, Leiderman and Katzman(9) demonstrated a significant difference in radio-potassium uptake by the brains of normal and adrenalectomized rats. They(8) found 20 mEq of radio-potassium per kilogram of wet brain to be non-exchangeable in normal adult rats, as determined by comparison of brain and plasma specific activities up to 72 hours after intraperitoneal injection. This non-exchangeable potassium compartment was not present in rats younger than 35 days and was abolished by adrenalectomy in adults.

Shaw and Simon(12) found that the potassium in toad nerve and muscle, allowed to die naturally or poisoned with iodoacetate or cyanide, did not equilibrate with the surrounding medium. They postulated potassium binding to explain this effect.

Shanes and Berman(11) studied diffusion out of toad sciatic nerve. They found that a small but significant proportion of radioactive sodium did not come to diffusion equilibrium with the surrounding medium.

Pappius and Elliott(10) noted that potassium content of rat cerebral cortex slices in a Warburg apparatus under anaerobic conditions failed to come to equilibrium with the medium when potassium concentration of the medium was high. They suggested that this disequilibrium might be due to binding of a large portion of intracellular potassium.

Recapitulation of the reported literature seems to indicate that there is probably

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"bound" potassium in brain tissue. The location or chemical moiety involved in the binding is suggested only by Folch's work, and the metabolic and/or chemical conditions of the binding are far from well-defined at this stage in our knowledge.

The term "binding" has been used by the investigators cited with several different though related meanings, with reference to observations that the electrolytes were: "retained by tissue," "combined with elements," "non-exchangeable," "slowly exchangeable," or perhaps "only present in unexpectedly high concentration." These differences in definition have probably accounted for most of the discrepancies reported by different workers.

The present study grew out of our previous study(5) of the diffusion of radioactive ions out of brain tissue slices. In this earlier paper, only the beginning of the diffusion process was considered. In the present investigation the later parts of the diffusion curves, at or near equilibrium, have been studied.

Radioactive tracers. Hypotonic or isotonic solutions of radioactive sodium ($Na^{24}Cl$) potassium ($K^{42}Cl$) or bromide (KBr^{82}) were used in these experiments, the first 2 as solutions in hydrochloric acid, the latter in water.

The solutions were brought to approximate neutrality prior to use. To adjust for their high radioactive concentrations, and for convenience in injection, the lots were diluted with normal saline.

General methods and tissue preparations. Unselected healthy adult male guinea pigs were employed in all experiments where brain tissue was required. In those experiments requiring radioactive brain tissue, each guinea pig was injected intraperitoneally with 200-400 μc of isotope solution, fed and watered *ad lib.*, and sacrificed after a delay to allow for distribution of the tracer: 12 hours for Na^{24} and 24 hours for K^{42} or Br^{82}. The uptakes of these tracers were determined in a series of experiments by sacrificing the animals 1½, 3, 6 or 9 hours after intraperitoneal injection.

All brain tissue was prepared as follows: The animal was rapidly decapitated, the brain was removed and divided longitudinally by a median cut through hemispheres and brain stem. Brain stem, basal ganglia, and choroid plexi were removed, then the pia was stripped with fine forceps, leaving the cortex and some white matter, but free of macroscopically obvious blood. At the same time, the liver was removed and small pieces cut from it. The strips of tissue were pressed firmly between sheets of absorbent paper to remove as much blood as possible. The tissue pieces were then transferred to a tared homogenizer tube and weighed. Standard mammalian Ringer's solution was added, and the mixture homogenized with a Potter-Elvehjem tissue grinder with Teflon pestle. Ringer's solution was added to the homogenate to bring the tissues to final dilution of 1:20.

Thoroughness of homogenization was evaluated by microscopic examination of the sedimented plug obtained by centrifuging samples of homogenate. Smears and paraffin sections of the sediment showed many intact nuclei but no intact cells. Irregular granular material with an occasional red cell or torn length of capillary were also noted.

The experimental procedures carried out were of two general categories: (1) equilibrium dialysis, and (2) centrifugation followed by analysis of the supernatant and sediment.

Dialysis experiments. For the dialysis experiments 20 ml of the dilute homogenate was dialyzed against 400 ml of isosmotic solution through cellophane sausage tubing. Dialysis was continued for 2 hours at room temperature.

Samples from the dialysis bag and the external solution were transferred to tared planchettes, weighed, and dried with a spreading agent. Radioactivity was determined either with a Scott type Geiger-Muller counter or a Tracerlab scintillation counter with 1.29 g/cm^2 lead shielding to remove beta emission.‡ Radioactive decay, self ab-

‡ We would like to thank Dr. K. G. Scott and staff of the Radioactivity Center, Univ. of California, San Francisco, for their very kind assistance.

sorption, and coincidence corrections were carried out as appropriate.

The materials to be dialyzed were prepared according to the following procedures.

Procedure 1: ("Blank" in Fig. 2). The dialysis bag was filled with a modified Ringer's solution to which was added a small drop of radioactive tracer, but no brain homogenate.

Procedure 2: ("Control" in Fig. 2). The dialysis bag was filled with homogenized non-radioactive brain to which was added a small drop of radioactive tracer. This procedure was the same as Procedure 1, except for addition of non-radioactive brain homogenate.

Procedure 3: ("Supernatant" in Fig. 2). The dialysis bag was filled with supernatant obtained by centrifugation of homogenized radioactive brain from a tracer-injected animal. See "Centrifugation Experiments."

Procedures 4 and 5: (NOT CONVULSED in Fig. 2). The dialysis bag was filled with homogenized radioactive brain from a tracer-injected animal, either not premedicated ("No Drug") or injected with sodium pentobarbital prior to sacrifice ("Pentobarbital").

Procedures 6-7-8: (CONVULSED in Fig. 2). The dialysis bag was filled with radioactive brain homogenate from a tracer-injected animal convulsed with one of 3 agents ("Cocaine," "Electric Shock," or "Metrazol"). The basic procedure here was the same as with Procedures 4 and 5 above.

An additional series of control dialyses was done with homogenized radioactive liver tissue from tracer-injected animals.

Centrifugation experiments. Centrifugation experiments were carried out on brain tissue from tracer-injected animals either after convulsions (cocaine, electric shock, or metrazol), or sacrificed without premedication. Thirty ml portions of dilute brain homogenate, prepared as described previously, were centrifuged for 15 minutes at 3,000 rpm. Samples of supernatant and sediment were weighed and dried, and radioactivity was determined. (Fig. 3). The supernatant was also studied by dialysis (see above, procedure 3).

Development of binding. A series of dialysis experiments were carried out with intervals of 1½, 3, 6, and 9 hours between injection and sacrifice, in order to define the rate at which the non-exchangeable electrolyte fractions develop. The technics were exactly the same as in the control dialysis studies, except for the variation in post-injection intervals.

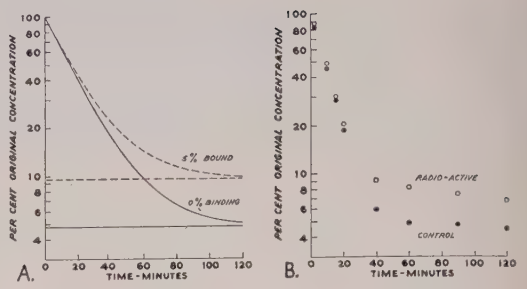
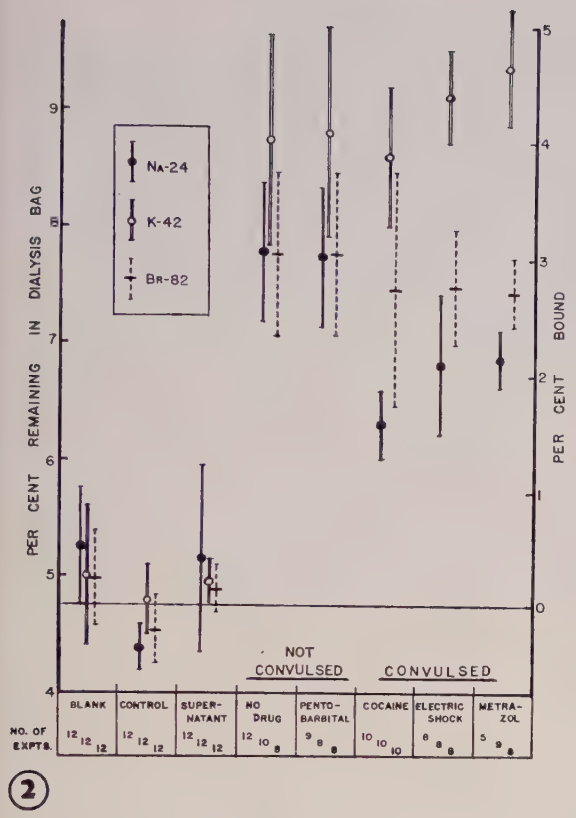


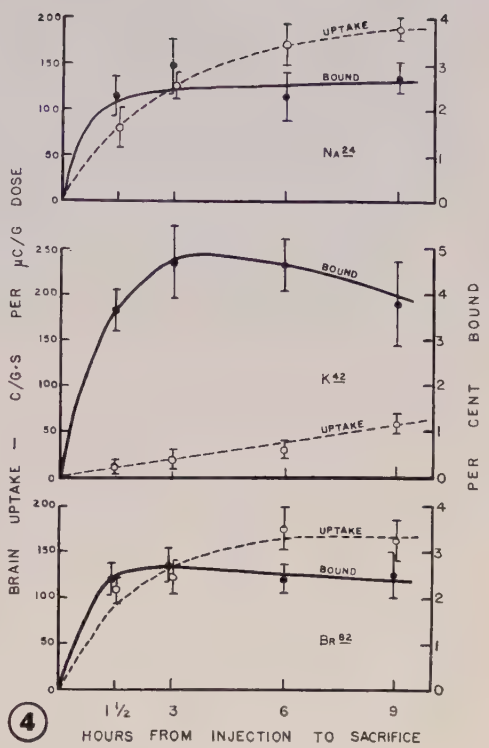
FIG. 1A. Theoretical dialysis curves based on experimental method described.

FIG. 1B. Experimental curves of the same type as theoretical curves of 1A, using radioactive bromide as tracer. Points indicate radioactivity found inside the dialysis bag after dialysis for various periods. See text for additional details.

Results. 1. *Dialysis experiments.* At equilibrium, assuming free permeability, radioactive tracer should be evenly distributed throughout the 20 cc inside the bag and the 400 cc outside the bag. Thus, if there is no fixation ("binding") of the radioactive tracer inside the dialysis bag, concentrations of both inner and outer solutions should be 4.76% of the concentration inside the bag at the beginning of dialysis, as in the lower theoretical curve of Fig. 1A. If 5% binding were present, the curve would approximate the upper theoretical curve of Fig. 1A. Fig. 1B shows the results of a series of preliminary experiments with radiobromide, which confirmed that binding actually was present. Similar results were obtained from preliminary studies with potassium and sodium. Comparison of columns 2 and 3 of Fig. 2 with column 1 immediately indicates the absence of significant binding of Na^{24}, K^{42} or Br^{82} *in vitro* or in the centrifuged supernatant. From the latter observation it appears that these isotopes are combined essentially, if not entirely, in the centrifugable portion of the homogenates (see also "Centrifugation Experiments").

It is to be emphasized that binding is re-





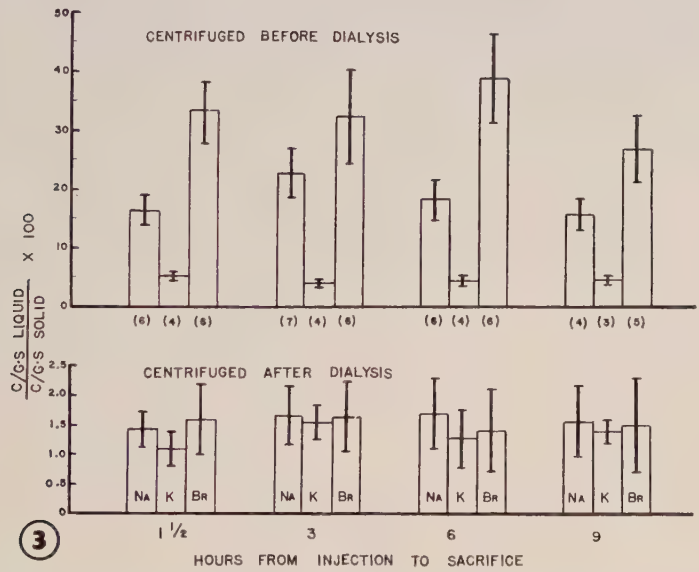


FIG. 2. Summary of dialysis experiments done under various control and experimental conditions. As described in text, values given for each set of data are recorded as percent of original radioactivity retained inside the cellophane dialysis bag at the end of dialysis (left scale) or percent of original brain radioactivity bound (right scale). Mean values are indicated, with stand. deviations.

FIG. 3. Centrifugation studies as described in the text. Means and stand. deviations are indicated. Parenthetical numbers represent numbers of experiments performed.

FIG. 4. Radioactive uptake and binding in the brain. The same animals were used for both determinations. Uptake, as indicated, was measured as proportion of inj. dose, while binding, as described elsewhere, was recorded as % of radioactivity originally present in the brain. Experimental points are means, and stand. deviations are indicated.

TABLE I. Effect of Convulsions on Binding. "Control" data are derived from columns 4 and 5 of Fig. 2, while "Convulsed" data derive from columns 6, 7, and 8. Values are reported as % of radioactivity present in the brain. Parenthetical numbers indicate number of experiments in the respective groups. Ranges given are stand. dev., and "p" was calculated using the "t" test.

	Controls, % bound	Convulsed, % bound	Significance of diff. "p"
Na^{24}	3.1 ± .6 (21)	2.0 ± .4 (23)	<.01
K^{42}	4.1 ± .9 (18)	4.3 ± .5 (27)	.4
Br^{82}	3.1 ± .7 (16)	2.8 ± .6 (26)	.1

ported here in percent of radioactivity *present in the brain.* There appears to be no obvious relation between these percentages and injected dose, at least in the dosage ranges employed here. Amount of binding can also be reported in mEq/kg wet brain in the case of sodium and potassium, but this is not possible or relevant with bromide, which is present only in tracer amounts.

The use of proportionality values (percentages) rather than chemical equivalents or other units appears to be justified by the observation that only such proportionality values make obvious the striking similarity between the binding of the 3 tracer elements studied, 2 of them normal brain constituents and the third a foreign tracer substance. No other investigator of whom we are aware has considered the possibility that such physiologically dissimilar substances might react in such similar ways in the brain. Possibly this has been missed because the similarities never appear with the usual methods of displaying the data.

Columns 4 and 5, Fig. 2, indicate that about 3% of sodium (1.7 mEq/kg wet brain), 3% of bromide, and 4% of potassium (3.5 mEq/kg) are bound *in vivo*, as measured by this technic.

Convulsions caused by cocaine, metrazol or electric shock (columns 6-7-8, Fig. 2) effected a significant decrease of the bound sodium: 50% (0.8 mEq/kg) in the case of cocaine, and 30% (0.5 mEq/kg) with metrazol or electric shock. No change in binding of potassium or bromide was noted in these convulsed animals. These data are summarized in Table I.

2. *Centrifugation experiments.* The results of the centrifugation experiments are given in Fig. 3. The guinea pigs used in these experiments were treated as described previously, samples of homogenate being taken for dialysis as well as for centrifugation. Centrifugation was carried out both before and after 2 hours' dialysis. The bars represent quotients obtained by dividing the calculated activity of the dry centrifuged sediment into the activity of the centrifuged supernatant. These quotients bear no straightforward relationship to percentage of isotope binding, but serve as a useful basis for comparison of different groups of experiments: the smaller the value of the quotient, the greater the proportion of bound isotope in the centrifuged sediment, and the less the proportion in the supernatant.

Analysis of the centrifuged homogenate before dialysis (Fig. 3) indicated that, of the 3 electrolytes, bromide was present in the sediment in smallest amount, sodium was next, and potassium was present in the greatest proportion.

After 2 hours of dialysis, on the other hand (Fig. 3, bottom), the proportions of sodium, potassium, and bromide in the sediment diminished, and became identical within the limits of experimental error.

3. *Development of binding.* The curves of Fig. 4 indicate that binding of all 3 ions studied occurred very rapidly, reaching nearly full value within 1½ hours after injection. This figure also shows the lack of relation between uptake and development of binding.

4. *Liver controls.* From every third or fourth animal a section of liver tissue was taken and dialyzed in precisely the same manner and at the same time as the brain tissue from the same animal. This was done as a further check on the method and to demonstrate possible differences between brain and a non-nervous tissue. Table II indicates that there is no significant binding of radio-sodium or bromide in liver homogenates. There is, however, questionably significant binding of K^{42} at about 40% the level observed in brain.

Discussion. The concept of binding is

TABLE II. Absence of Binding in Homogenized Liver. Ranges given are stand. dev.; "p" values were calculated by "t" test.

	% remaining in dialysis bag at 2 hr	Apparent % bound	Significance of binding, "p"
Na^{24}	5.6 ± 1.3 (12)	.9	.4
K^{42}	6.5 ± 1.0 (12)	1.8	.1
Br^{82}	5.1 ± .8 (14)	.5	.4

rather inexact at times, and criticism can justifiably be leveled against any approach so far employed in study of the subject. The major methods which have been used in the past are: 1. Analysis of tissue extractives, 2. Diffusion procedures employing semipermeable membranes, 3. Centrifugation of tissue homogenates, 4. Measurement of ionic activities by physical or chemical methods (*e.g.,* membrane electrodes), 5. Influx or outflux studies, and 6. Nuclear resonance technic.

In the present experiments, methods 2 and 3 were used. Demonstration that a tissue extractive contains a certain amount of an ionic species does not prove that this combination is physiologically important, nor that there is any actual combination whatsoever *in vivo*. Similarly, failure to show a bound ionic fraction by a dialysis method does not negate the possibility that there may be in the intact cell a loose combination of the ion, of great physiological importance.

Not only must the rate at which an ion complex can dissociate be considered, but also the site of binding within the cell may be of basic importance. Bartley and Davies (1) showed that mitochondria could maintain concentration gradients of sodium, potassium, magnesium, and phosphate under a variety of conditions. They obtained maximum ratios of internal concentration to external concentration at 0°C in absence of substrate enzymatic co-factors, such as ATP or DPN, when concentration in the external medium was very low.

Our observations for Na^{24}, K^{42} and Br^{82} suggest that the bound fractions are not present in mitochondrial or microsomal fractions, as defined by other workers. The centrifugation methods used here would leave these fractions in the supernatant, and no binding was observed in these centrifuged supernatants. (Cf Fig. 2, third column).

Age may be a significant variable, as indicated by Katzman and Leiderman's(8) demonstration of a non-exchangeable potassium compartment in rat brain, developing with age. We have carried out studies on the effect of age on binding as we have defined it, which will be reported later.

Our results are in contrast with those of Bergen, Stone, and Hoagland(2), who used a dialysis technic similar to ours and found about 30% of adult rat brain K^{42} to be non-diffusible through cellophane. The reason or reasons for this difference in results are not presently clear to us. Degree of homogenization and possible species differences remain to be investigated.

The absence of binding of sodium in guinea pig liver is in partial disagreement with data of Griswold and Pace(6) on rat liver. They found, using flame photometry following perfusion and differential centrifugation, that sodium and potassium were retained (7%-18%) in "mitochondrial" and "microsomal" fractions of minced liver.

The effect of convulsions on bound Na^{24} is most interesting. The decrease in the bound sodium (Fig. 2) must represent an actual loss from the bound compartment during convulsions, rather than a simple dilution effect such as might result from development of brain edema, since if the 50% change were a simple dilution effect, total brain sodium would have had to rise to the 200% level, which was impossible under these experimental conditions.

There was no significant increase or decrease in the binding of potassium or bromide during convulsions. This lack of change may mean that potassium and bromide are more strongly bound than sodium. More likely, however, it simply implies that any changes which occur effect both bound and unbound fractions similarly, so that the 2 remain in equilibrium and the relative proportions do not change.

The results of experiments on the dialysis of centrifuged supernatant and the studies on pre- and post-dialysis centrifugation support each other in demonstrating that the

bound fractions are in the solid, sedimentable component of the brain homogenate. These observations, the similar percentages of binding of the 3 elements, the similarity of the time courses of development of their binding in the brain, and the lack of binding in liver constitute considerable evidence of parallel behavior of physiologically dissimilar ionic species (Fig. 2, bottom of Fig. 3, and binding curves of Fig. 4). These data suggest that the binding may depend upon some unique characteristic of brain tissue, rather than being a function of the specific physiological characteristics of the individual elements.

Myelin is the most obvious unique constituent of brain. It has been demonstrated by electron micrography to have a layered structure. We should like to suggest that the occurrence of similar proportions of binding of the 3 dissimilar elements studied here may depend upon the colloidal nature of the myelin membrane. On the other hand, the diminution of binding of only one of these elements (sodium) as a result of convulsive activity suggests that the quantitatively similar binding of the 3 may be coincidental.

Further studies of a more specifically chemical nature are presently under way to evaluate these possibilities.

Summary. 1. Dialysis of guinea pig brain homogenates from animals given intraperitoneal injections of radioactive tracers revealed that 3%-4% of brain sodium (1.7 mEq/kg wet brain), bromide and potassium (3.5 mEq/kg) were "bound", *i.e.*, did not pass the dialysis membrane. 2. Centrifugation studies indicated that these "bound" fractions were contained in the sedimentable portion of the homogenates, and therefore are not mitochondrial or microsomal, as these terms are usually defined. 3. Similarities in amount and development of "binding" of these 3 elements, and in the effects of centrifugation, as well as the absence of comparable binding in liver suggested that this binding may be related to the presence of myelin. 4. The level of bound sodium decreased 50% as a result of convulsions, with no concomitant change in bromide or potassium. 5. The significance of these observations was discussed.

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Myocardial Depletion of Norepinephrine in Hemorrhagic Hypotension.* (26746)

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In shock, elevated plasma levels of catecholamines(1,2) were primarily found to result from a highly augmented secretion from the adrenal medulla(3,4). These observations prompted us to explore whether changes in plasma catecholamine levels would induce an alteration in myocardial concentration of epinephrine and norepinephrine. We assumed that these observations would be significant, since recent observations have correlated myocardial catecholamines with cardiac performance(5,6).

Methods. Analysis of myocardial epinephrine and norepinephrine were conducted on 2 groups of albino rabbits ranging in weight from 1 to 3 kg. One group comprising 12 normal rabbits were killed by a blow on the head. The heart was removed and a sample of left ventricle was weighed after the muscle was washed with 0.9% NaCl and dried on filter paper. The muscle was homogenized in 10% trichloroacetic acid in a Potter-Elvehjem tissue grinder, immersed in ice and water. The supernatant liquid, after refrigerated centrifugation, was extracted with diethyl ether. The aqueous portion was diluted with an equal volume of 0.2 N sodium acetate, and the pH adjusted to 8.2 with 0.5 N Na_2CO_3. The tissue extract was passed through an alumina (Fischer) column, which had previously been washed 3 times with tripled distilled water. Eluates from columns were analyzed according to a modification of the trihydroxyindole fluorescence method(7) with epinephrine and norepinephrine standards. Reagent blanks and tissue blanks represented approximately 15% of the total fluorescence. Differentiation of epinephrine from norepinephrine was based on using Corning exciting filters of 400 mμ and 440 mμ with Wratten Filter 35. A Corning 500 mμ filter with a Wratten Filter 57 was used for emission. Because of low transmission, interference filters for greater spectral purity were not employed. The sensitivity of our instrument was arbitrarily adjusted to detect accurately 0.01 to 1.5 μg of epinephrine and norepinephrine. Ether extraction of homogenized heart tissue was found to increase fluorescence by 8%. Our recoveries from adding catecholamines to the alumina column averaged 85%. In those steps involving the analysis of eluates, recovery of added amounts to 10 samples ranged 88 to 110%. The overall recovery for all steps of the catecholamine analysis averaged 82%.

The cardiac catecholamines from the control group were compared with those found in 12 rabbits subjected to hemorrhagic hypotension for 3 hours at mean arterial blood pressures of 50 mm Hg. Under local anesthesia, the femoral artery of a hind limb was catheterized for bleeding and for maintaining mean blood pressure. Blood pressure was maintained constant by elevating a graduated cylinder containing the initial bleeding volume (av. 30 ml/kg) to a height equivalent to 50 mm Hg pressure. Near the end of the hypotensive period, 25 ml of blood was drawn from the artery for plasma catecholamines analysis in 4 of 12 rabbits. The heart was taken out for analysis after the animal was killed by rapidly removing with a large syringe, the remaining blood volume. An initial dose of 5 mg/kg of Heparin‡ was administered as an anticoagulant, ½ of the initial dose was repeated every hour. Peripheral plasma catecholamine levels were studied in 4 normal rabbits from blood drawn by cardiac puncture.

Results. A striking difference in myocardial content of catecholamines was found when normal rabbits were compared to rabbits subjected to hemorrhagic hypotension

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TABLE I. Myocardial Norepinephrine and Epinephrine in Normal and Hypotensive Rabbits.

	No. of rabbits	Control (μg/g)	Hypotension (μg/g)	% diff.	P†
Norepinephrine	12	1.05 ± .13*	.16 ± .05	−84.8	<.001
Epinephrine	12	.14 ± .06	.24 ± .06	+71.5	<.01

* Mean ± stand. error.

† Calculated by Student's "t" test.

for 3 hours. The summary of data in Table I shows that a marked depletion of norepinephrine (NE) occurred in the hypotensive group. Control levels averaging 1.05 ± .13 μg/g were reduced to 0.16 ± .05 μg/g, a reduction of 85%. This depletion of NE occurred concomitantly with a significant increase in epinephrine (E), where control levels of 0.14 ± .06 μg/g were elevated to 0.24 ± .06 μg/g, an increase of 72%. Despite the relatively high percentage increase in myocardial E, total catecholamine content (NE and E) of left ventricle was reduced to 34% of the normal rabbit heart.

In 4 normal rabbits, plasma E and NE were present in concentrations below the sensitivity of analysis (<.01 μg/15 ml). Plasma analysis in 4 of 12 rabbits with hemorrhagic hypotension averaged 1.4 μg/100 ml for E and 0.2 μg/100 ml for NE. The elevated myocardial content of epinephrine was probably due to its high concentration in circulating blood(8).

In Table II are shown myocardial NE levels in 4 additional hypotensive rabbits. These animals were treated, after a 3 hour period of hemorrhagic hypotension at 50 mm arterial pressure with varying doses of NE (Levophed base). Thirty minutes later, the hearts from these rabbits were removed for NE analysis after rapid exsanguination. The data indicate a good dose-uptake relationship between NE dose and level found in left ventricle. Levels of myocardial NE were also found to be related to the level present in plasma (Table II). The most significant point which can be made for these uptake experiments is that myocardial depletion of NE in the hemorrhagic hypotensive animal is reversible. It also follows that the extent to which the myocardium will store exogenous NE depends on the dose administered, thus indicating that its passage into the muscle cell may simply be governed by diffusion rather than an active process.

TABLE II. Myocardial Uptake of Norepinephrine in Hemorrhagic Hypotension.

Rabbit No.	Dose (μg/kg)	Heart (μg/g)	Plasma (μg/100 ml)
1	500	1.59	1.90
2	100	.93	1.10
3	50	.85	.43
4	25	.65	.21

Discussion. Current reports have focused attention on the role of NE from sympathetic postganglionic nerve endings on myocardial function. Sarnoff(6) considers the "catecholamine stimulus" on ventricular contractility equal in importance to the Frank-Starling relationship of end-diastolic pressure and fiber length. Mayer and Moran(9) reported that injected catecholamines augmented myocardial phosphorylase activity, which in turn was found related to contractile force of the heart *in situ*. Indirectly, function of the adrenergic transmitter has also been ascertained from studies involving its depletion by cardiac sympathectomy or pharmacological agents. These studies show the depleted heart to have less force of contraction, a slower heart rate, and a decrease in right atrial pressure(10,11). In view of these findings, our results on myocardial depletion of NE indicate that failure of this organ to maintain circulatory homeostasis in a progressing state of shock will be severely limited. The experimental design of the reported experiments does not permit us to explain the underlying mechanism of myocardial depression in near terminal hemorrhagic shock(12). However, our data suggest that genesis of myocardial failure in this state could be due to NE depletion.

Gilmore *et al.*(13), and Fozzard and Gilmore(14) reported that treatment of animals in hemorrhagic shock with NE will prolong survival. Lansing and Stevenson(15) found

that administration of NE in shock prevented decreases in cardiac output, which occurred in the non-treated animals with the progression of shock. Caliva *et al.*(16) using an electropolarographic technic, observed that NE in early shock will restore normal myocardial oxygen levels. These investigators proposed that the prolonged survival period of NE-treated animals was due to a cardiotonic-like action of the hormone. In accordance with our results, the increased survival time may have arisen from the uptake of administered NE by the depleted myocardium. Replenishment of the adrenergic transmitter could have sustained cardiac contractility, thus preventing further decline in cardiac output. On the other hand, some laboratories have noted no change in the steady downhill course of NE-treated animals in hemorrhagic shock(17,18). The current disagreement on the therapeutic value of NE probably arises in comparing experimental results from animals in which NE was injected at different times during the course of shock. It is highly probable that the reported ineffectiveness of this catecholamine in prolonged shock is due to the entry of other factors responsible for its fatal outcome.

Summary. Analysis of NE and E in the left ventricle of 12 normal albino rabbits averaged 1.05 ± .13 and 0.14 ± .06 μg/g respectively. In 12 rabbits subjected to hemorrhagic hypotension for 3 hours at mean arterial blood pressures of 50 mm Hg, myocardial NE declined significantly to an average of 0.16 ± .05 μg/g ($P<.001$). Epinephrine levels in this group rose to an average of 0.24 ± .06 μg/g ($P<.01$). Injection of graded doses (500 to 25 μg/kg) of NE in hypotensive rabbits caused an elevation of myocardial NE in rough proportion to the dose administered and level in plasma.

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Antigenicity of Short Ragweed Pollen for White Mice.* (26747)

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In the course of our early studies on enhancement of anaphylactic sensitivity by *Bordetella pertussis* it was found that proteins differed in their ability to sensitize(1-4). Included in the group was extract of

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TABLE I. Effect of *B. pertussis* on Sensitization by Short Ragweed Pollen.

Challenge dose		Sensitizing dose						
Ragweed	Day	Control	*B. pertussis*	WSR 5 mg	*B. pertussis* 0.5 ml 10% aq.	*B. pertussis* 5 mg WSR	*B. pertussis* 5 mg WSR	Adjuvant 5 mg WSR
0.4 ml 10% aq.	4	0/4	0/11					
5 mg WSR	5	0/10	0/10					
0.1 ml 10% aq.	10		↓		0/16			
5 mg WSR			reinject	0/10			1/10	
5 mg WSR	15		0/10	↓ reinject			↓ reinject	
Idem	17		↓ reinject	↓ 0/10		3/10	↓ 8/9	2/10
"	22		↓ 0/10					

Ambrosia elator pollen, the short ragweed, which was found to be of limited antigenicity by us(5) as well as by Bukantz and Johnson (6). The recent literature(7,8) has indicated, however, that extracts of this and other pollens are capable of exciting an endotoxic-like effect in the pertussis-treated mouse. Since previous observations failed to indicate such an action, the question of the antigenicity of short ragweed pollen extract was reconsidered.

Materials and methods. Swiss-Webster female mice weighing about 20 g at start of the experiment were inoculated intraperitoneally with 0.5 ml of pertussis vaccine containing about 6×10^9 phase I organisms. Two preparations of short ragweed pollen were employed; a 10% (w/v) aqueous extract (AE) or the WSR fraction prepared according to the method of Sehon *et al.*(9). Technics consisted of incorporating AE or WSR together with the pertussis vaccine in the initial inoculation followed by an intravenous challenge by the homologous allergen on the 10th and/or a subsequent day following sensitization. In addition, sensitization by WSR alone and in Freund's complete adjuvant(10) was attempted. Finally, the effect of WSR in mice previously treated with pertussis vaccine was noted.

Results. The following observations which are apparent from the data in Table I were made: 1. Neither AE nor WSR was lethal to mice previously untreated. 2. Neither AE nor WSR was lethal to mice treated 4 or 5 days previously with *B. pertussis*. A subsequent inoculation with WSR was also without effect. 3. Repeated injections of WSR into mice on the 5th, 15th, and 22nd day subsequent to *B. pertussis* failed to be lethal. 4. Incorporation of AE or WSR into the initial sensitizing dose of *B. pertussis* failed to enhance anaphylactic sensitivity when the subsequent challenge was made on the 10th day, death being the criterion. With challenge by WSR on the 17th day subsequent to pertussis-inoculation, 3/10 deaths were noted. This compares with 2/10 deaths obtained on the 17th day by challenge of Freund's adjuvant-treated mice. 5. A striking secondary response was observed when mice were challenged with WSR on day 10 (1/10 deaths) and rechallenged on day 17 (8/9 deaths) following sensitization with pertussis-WSR.

Discussion. In agreement with previously unreported results(5,6) the aqueous extract of short ragweed pollen was found to be a poor antigen for mice since no enhancement of anaphylactic sensitivity was obtained by using the pertussis-technic(1). The WSR fraction was also shown to be relatively devoid of antigenic action. However, as has been observed for other systems, a striking secondary response is apparent following a repeated challenge. The relationship of circulating antibody to anaphylaxis is under study.

Contrary to the observations of Kind(7,8) for short ragweed and other pollens no endotoxic-like responses were obtained in the mouse previously treated with *B. pertussis*.

Although it is known that mice so treated show an enhanced susceptibility to many pharmacological agents, histamine, serotonin, Proteose-Peptone, etc., the mechanism involved in probably specific; for other agents, compound 48/80, epinephrine, Bacto-Peptone, etc., are without effect.

Summary. Short ragweed pollen extract is shown to be a poor antigen for mice even with recourse to the pertussis-technic. A subsequent challenge dose demonstrates a striking secondary response. Extracts fail to display any lethal effects in mice previously treated with *B. pertussis*.

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Precipitating Antibody in Normal Human Urine.* (26748)

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Agglutinating antibody has been demonstrated in human urine against several organisms(1-5). Significant amounts of globulins have been found in normal human urine, and some of them were shown to be immunologically and electrophoretically similar to those of the serum(6-10). The present paper reports the demonstration of precipitating antibodies in normal urine.

Materials and methods. Patients. Two groups of patients with normal blood urea nitrogen and urine that was free of protein by the sulfosalicylic acid test were chosen for these studies. In the first group, 4 patients who gave a negative Schick test but whose serum failed to react with diphtheria toxoid were injected with 1.0 ml of purified diphtheria toxoid‡ subcutaneously; specimens of serum and 24-hour collections of urine were obtained at the end of the second and third weeks after the vaccination. The second group consisted of 11 patients convalescing from pneumococcal pneumonia; serums from these patients were tested for precipitins using as antigen the supernatant of a 5-day culture of the pneumococcus isolated from their own sputum or blood. Urine was collected over a period of 48 hours from each of those patients whose serum gave a precipitate in the ring test.

Urine. The urines were collected in clean containers without preservative. Each specimen was filtered through Whatman #12 filter paper and the filtrate was dialysed against running tap water at 4°C for 24-48 hours. Comparisons were first made of the efficacy of several concentration procedures which included: evaporation under negative pressure, pervaporation at 4°C and at room temperature, ultrafiltration(11), ammonium sulfate precipitation(10), and lyophilization. The following method was chosen as the most efficient for the purpose at hand: Immediately following dialysis the urines were concentrated at room temperature by pervapora-

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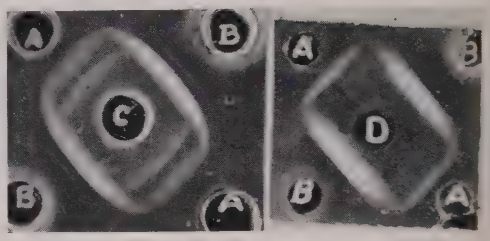
‡ 50 Lf units/ml; obtained from Div. of Biological Labs., Mass. Dept. Public Health.

tion. By using Visking tubing (inflated diameter 20/32 in., wall thickness 0.0008 in.) and large portable fans in a walled-off enclosure, a 24-hour output of urine could be reduced to approximately 50 ml within 8 hours. The concentrated urine was then lyophilized and the resulting dry powder was suspended in 2 to 4 ml of barbital buffer, pH 8.6. The preparation was kept at 4°C overnight, centrifuged, and the clear supernate was used. By this technic, a 24-hour sample of urine could be processed completely within 48 to 72 hours.

Immunological technics. Double diffusion in Agar gel. A modification of the Ouchterlony technic described by Grasset *et al.*(12) was employed. Glass slides, 25 × 75 mm, were overlaid with 4 ml of 2% agar (Difco). The wells were formed with Pasteur pipettes into which agar plugs were drawn up with a rubber bulb attached to the free end: these wells could hold from 0.01 to 0.03 ml of the substance to be tested. After filling the wells, the slides were placed in Petri dishes, the bottoms of which were covered with wet filter paper. The dishes were covered and sealed with vaseline to maintain a high humidity and were kept at room temperature. Depending on the concentrations of the constituents being tested, precipitin lines appeared between 4 and 12 hours. When urine was used as an antibody preparation, up to 48 or 72 hours were necessary for complete development of the precipitin lines and it was often necessary to refill that well several times during the first day.

Immunoelectrophoresis. A modification of the method described by Williams and Grabar(13) was used. The Spinco Model R cell operated with a Heathkit variable voltage regulated power supply, Model PS-3 was employed. With this system, 5 large (50 × 75 mm) or 13 small (25 × 75 mm) slides could be run in parallel. The gradient across each slide was maintained at 40 volts, a 2 to 3 hour run producing satisfactory separation of the proteins. Large slides were overlaid with 9 ml of 2% agar prepared by adding equal volumes of barbital buffer (pH 8.6 and ionic strength 0.075) and 4% agar (Difco) in distilled water. Wells, similar to those described above, were cut out of the agar for the substance to be electrophoresed. Following electrophoresis, longitudinal basins were cut out, with razor blades, parallel to the direction of electrical flow; these were filled and the slides placed in a humid atmosphere at room temperature. The precipitin pattern was usually established after 24 hours of diffusion and was completed in 2 or 3 days. Photographs of unstained slides were made with a modified dark field microscopy technic(14).§ In some instances it was found necessary to stain the precipitin lines for better photographic representation; in that event, the preparations were first washed in several changes of physiologic saline for 2 days. After staining with either Ponceau S or Nigrosin (Consolidated Laboratories, Inc., Chicago Heights, Ill.) the slides were dried at 37°C for permanent mounting.



(a) (b)

FIG. 1. Double diffusion in agar of urine (A) and serum (B) with rabbit anti-human globulin (C) and diphtheria toxoid (D). Patient was immunized with diphtheria toxoid.

The modification of Wadsworth and Hanson(15) was used to demonstrate the presence of precipitates not observed with the standard immunoelectrophoresis. This involved diffusion of the separated material from an additional basin to increase its concentration above the threshold for formation of a visible precipitate.

Results. Diphtheria. The specimens of concentrated urine from 3 of the 4 patients immunized with diphtheria toxoid reacted with that antigen. The serum of each of these 3 patients formed very clear and dense precipitates, whereas only a weak precipitin line resulted from the reaction of the fourth patient's serum and the toxoid. Fig. 1a il-

§ By Mr. Leo Goodman, Mallory Inst. of Pathology.

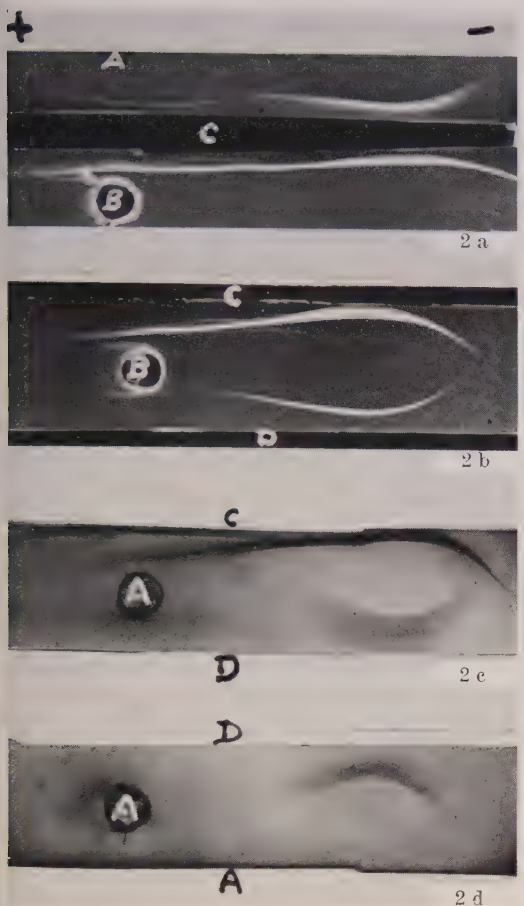


FIG. 2. Immunoelectrophoresis of serum and urine of same patient as in Fig. 1. A = urine; B = serum; C = rabbit anti-human globulin; D = diphtheria toxoid.

lustrates the precipitin lines obtained when a urine preparation and serum from one of these patients were reacted with an anti-human-γ-globulin.¶ The line closest to the urine well showed a reaction of complete identity with the major line formed by the patient's serum. These results are similar to those of Franklin(6) who reacted urine γ-globulin and serum 7S globulin in agar with an antiserum prepared against Cohn Fraction II; 2 or 3 lines were usually present in the case of the γ-globulins prepared from urine and the line closest to the antigen well showed a reaction of identity with that formed by the 7S γ-globulin. Fig. 1b shows the reaction of complete identity produced by the same serum and urine with diphtheria toxoid.

Fig. 2 is a composite of the results obtained by immunoelectrophoresis with the same serum and urine. Fig. 2a shows the similar electrophoretic mobilities of the γ-globulin fractions of serum and urine, and Fig. 2b shows that the antibodies to diphtheria toxoid reside in the γ-globulin fraction of the serum. The precipitate formed by the interaction of urinary antibody and diphtheria toxoid is seen in Fig. 2c in the area corresponding to the electrophoresed γ-globulin of normal serum. Some difficulty was encountered in arranging the optimum proportions of diphtheria toxoid (antigen) and urine (antibody) to form a clear precipitate. The reinforcement technic(15) produced a more satisfactory precipitin line for photographic purposes as shown in Fig. 2d. When the longitudinal basins were arranged to form a rectangle around the separated urine and serum proteins, as suggested by Grant(9), the γ-globulins of serum and urine were found to join, showing, as with ordinary agar diffusion, that they are identical antigenically. Urine and serum collected from 3 Schick positive patients did not react with the diphtheria toxoid.

Pneumococcus. Of the 11 patients convalescent from pneumonia, the serum of 6 showed a visible precipitate by the ring test. By capsular swelling, the pneumococci from 2 of them were identified as type 3, in 2 others as type 4, in 1 as type 7, and in 1 the pneumococcus reacted with a pool of antisera of various types and was not specifically identified. The samples of serum from each of these 6 patients gave precipitin lines with the crude pneumococcal antigen preparation (culture supernates) in the micro-Ouchterlony technic. However, only the urine of the 2 patients convalescing from type 4 infection, and of one who recovered from type 3 pneumonia gave precipitin lines when reacted against the supernatant of a culture of their

¶ The antisera to human γ-globulin (Cohn Fraction II, provided by Protein Foundation, Jamaica Plain, Mass.) were prepared in rabbits by subcutaneous and intramuscular injections with Freund adjuvant and supplied by Dr. Ramzi Cotran, Mallory Inst. of Pathology.

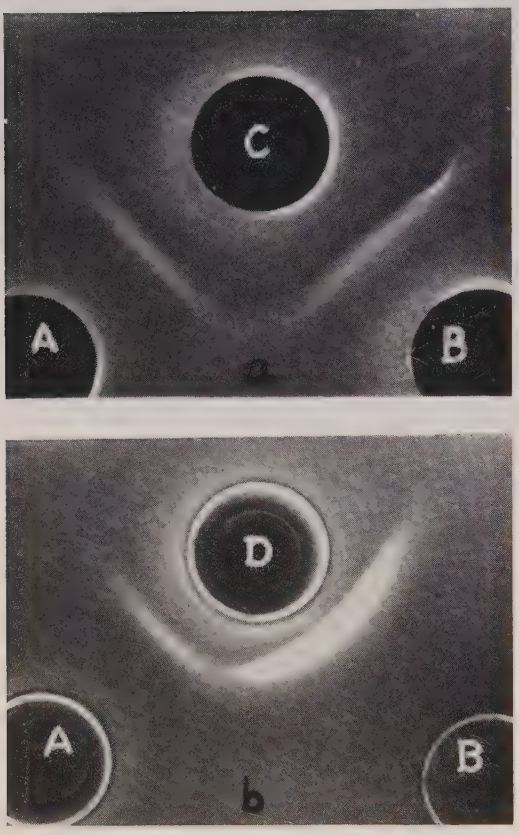


FIG. 3. Double diffusion in agar of urine (A) and serum (B) with culture supernate (C) of the pneumococcus isolated from the patient and with capsular polysaccharide (D) of the same type. The patient was convalescing from type 4 pneumococcal pneumonia.

own organism. An example is given in Fig. 3a.

The specimens of serum and urine which precipitated with their respective culture supernates also reacted with the corresponding purified type-specific pneumococcal polysaccharide.‖ This is illustrated in Fig. 3b. No reaction was discerned between any patient's serum or urine and any culture supernate or specific polysaccharide of a different pneumococcal type.

Discussion. In this study, although precipitating antibody could be demonstrated in the serum of each patient, it was not demonstrable in the urine of some of them by the technics used. Since total daily output of urinary protein varies widely(8), concentration of larger volumes from the other patients might have produced positive results. If the antibody found in urine is derived from the serum, a threshold level may be necessary in the serum for it to be excreted or to become demonstrable in the urine(16). The results of the present study are only qualitative and no attempt at quantitation was made.

Several studies of urinary antibody to specific bacteria have been reported. Burrows and Havens(1) demonstrated agglutinating antibodies to cholera vibrio and to typhoid bacilli in the urine of normal volunteers immunized with the corresponding vaccines. The cholera antibodies seemed to appear and disappear in the urine independently of the levels in the serum, peak titers occurring in the former 16 to 19 days and in the latter 26 to 42 days after inoculation. Unconcentrated urine was used in those studies, but the agglutinins to *V. cholerae* were also demonstrated in globulin isolated from the urine.

Naylor and Caldwell(4), working in Egypt with patients who had urinary shistosomiasis and also were urinary carriers of Salmonella, presented data which they interpreted as indicating that the urinary antibody was derived in part from serum but was also formed locally within the urinary tract. Their patients, like those of Archer, *et al.* in another study(5), had gross proteinuria, so that the results cannot be compared with those of the present study which was done in patients with normal urine; in the urine of such patients they failed to demonstrate flagellar agglutinins after TAB vaccination.

Agglutinating antibody to Leptospira has been found in the urine of patients during acute and convalescent stages of infection with this organism(2,3). The urines of a few of the patients' studies by Stuart(2) were negative for protein when examined by the sulfosalicylic acid test, and the agglutination was demonstrated in voided specimens without prior concentration. Stuart considered urinary antibody to Leptospira to be of considerable diagnostic value because of its re-

‖ The type specific polysaccharides were supplied in 1935 by Dr. Rachel Brown, Bureau of Laboratories, New York State Dept. of Health; the type specific antisera were provided by Lederle Laboratories.

markably greater species specificity when compared with serum of the same patients.

The normal daily urinary excretion of protein varies widely in different individuals and may be as high as 150 mg in 24 hours(6,8,10, 17,18). Rigas and Heller(8) found the mean normal excretion in 24 hours of protein to be 39.0 mg in 17 normal subjects. The average for albumin and globulin was 14.8 mg and 25.8 mg respectively, with an average A/G ratio of 0.51, the reverse of that found in normal serum. In the present study the total protein in the 24-hour urinary output of the 11 patients studied averaged 43.5 mg (range 23-60 mg) by the Biuret method(19).

Electrophoretic analysis of the proteins present in normal urine revealed mobilities corresponding to those of the 5 electrophoretically distinguishable components of normal serum(8). Certain of the physicochemical and immunochemical properties of the biocolloids (of normal urine) have been characterized by Webb, Rose, and Sehon(7) and by Franklin(6). They demonstrated urinary proteins which were antigenically closely related to serum γ-globulins but which differed from them in being smaller in size. Molecular weights of these globulins ranged from 10,600(7) to 35,000(6). Franklin reported a sedimentation constant of approximately 1.6 S for the major constituent of the γ-globulin fraction of normal human urine and demonstrated, by isotopic studies, that this constituent is derived primarily from the serum γ-globulin fraction. An additional component corresponding to the 7S γ-globulin of serum was also demonstrated but never exceeded 10% of total urinary protein. This more rapidly sedimenting component increased in absolute and relative concentration and constituted more than 75% of the total γ-globulin fraction in 5 patients whom they studied; these patients who had pyelonephritis, glomerulonephritis, or nephrosclerosis excreted 5 to 10 g of urinary protein per day.

Isotopic studies(6,18) add support to the hypothesis that urinary γ-globulins are derived from the corresponding serum fractions. However, Franklin was not able to demonstrate the presence of the low molecular weight globulins in the sera of patients with renal shutdown in whom an accumulation of these proteins might be expected. It is not clear whether these small globulins are products of breakdown or stages in the synthesis of the normal serum components and also whether they are added to the urine after completion of glomerular filtration or originate in the tissues of the urinary tract(6,18).

Since 7S γ-globulin is present in normal human urine it is probable that the antibodies demonstrated in the present study reside in that fraction. However, Porter's isolation of biologically active fragments with sedimentation coefficients of about 3.5S and molecular weights of 50,000 to 80,000 from a papain digest of rabbit γ-globulin antibody suggests that the small globulins in normal urine may act as antibodies(20).

The biological significance of the urinary antibodies is not clear. Since the antibodies in the urine of healthy persons are constantly diluted by the large amounts of urine, a marked protective effect locally seems unlikely. It is conceivable, however, that even such small amounts of specific antibodies in the urine might interfere with the isolation of certain pathogens, *e.g.,* Leptospira(2), viruses(16), by their direct effect on the organisms or by acting as opsonins. In the presence of gross proteinuria such as might accompany a febrile illness, such an effect may be even more likely.

Summary. The presence of precipitating antibody was demonstrated to diphtheria toxoid in the urine of patients immunized with that antigen and to pneumococcal polysaccharide in the urine of patients convalescing from pneumococcal pneumonia.

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Virological Studies on Acute Respiratory Disease in Young Adults. I. Isolation of ECHO 28.* (26749)

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The JH and 2060 viruses were originally isolated from cases of mild acute respiratory disease and have been reported to induce this type of illness in human volunteers(1,2,3,4). Recent laboratory investigations have failed to detect any significant antigenic differences between these 2 viruses(5,6), which have now been designated ECHO 28 by the Committee on ECHO Viruses(6). No additional isolations of ECHO 28 have been reported and its incidence has been deduced entirely by serological methods(5,7). The present report describes the isolation of 8 strains of ECHO 28 from young adults with naturally occurring mild acute respiratory infections.

Materials and methods. Study population. One hundred and one freshmen and sophomore medical students, 5 of whom were women, were enrolled as volunteers in this project. The range in age was from 20 to 31 years. At approximately 6-week intervals throughout the academic year the total group was sampled as described below and served as asymptomatic controls. Each individual reported to the laboratory at the very first signs of acute respiratory disease, when additional samples were taken. They returned 2 to 3 weeks later for convalescent specimens.

Samples. Separate dry throat and nose swabs were taken in duplicate. One set was placed in 0.5% bovine serum albumin in phosphate buffered saline for virus isolation. The second nose and throat swabs were placed in individual tubes of broth for bacteriologic studies. A portion of the samples for viral study were inoculated into tissue cultures on the same day, and the remainder was frozen at –40°C. Serum specimens were also obtained and stored at –20°C.

Tissue cultures. The H.Ep. 2 line was carried in our laboratory on Eagle's medium (MEM) with 10% calf serum. Cultures used for virus isolation were maintained on Eagle's medium with 5% inactivated chicken serum and were incubated in stationary slanted racks at 37°C for at least 14 days after inoculation. Monkey kidney tissue cultures were prepared from cells that had been grown in bottles, trypsinized and frozen according to the method of Stulberg *et al.*(8). The cell suspension, stored at –90°C, was quickly thawed and diluted in growth medium containing 0.5% lactalbumin, 2% calf serum and 0.2% SV-5 rabbit antiserum to provide cultures with islands rather than a complete monolayer of cells. These cultures were maintained on 50% Medium 199 and

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50% Eagle's medium with 0.2% SV-5 antiserum.

Human kidney cultures were prepared from cell suspensions preserved in the same manner as the monkey kidney cells. These cultures were grown on medium described by Hsiung(9) and maintained on 50% Medium 199, 50% Eagle's medium with 1% calf serum. The maintenance medium for both monkey kidney and human kidney contained .03% sodium bicarbonate. To all media were added 100 units/ml penicillin, 100 mg/ml streptomycin and 1 mg/ml amphotericin B. After inoculation with specimens, both the monkey kidney and the human kidney cultures were incubated at 33°C on roller drums. Tissue cultures were examined 3 times a week and maintenance medium was changed twice a week. Monkey kidney cultures were tested for the presence of hemadsorbing viruses by the method of Chanock *et al.*(10) at 3 to 5 day intervals during the 3-week incubation period.

Titration of viruses. The JH and 2060 viruses were obtained from Dr. W. J. Mogabgab, Tulane University, New Orleans, La. Titrations were carried out by inoculating into each of 2 tubes of appropriate tissue culture 0.1 ml of 10-fold falling dilutions of virus prepared in 0.5% bovine serum albumin in phosphate buffered saline. The endpoint was considered as the highest dilution producing a cytopathic effect involving at least 20% of the cell culture within one week following incubation.

Neutralization tests were carried out by a modification of the method described by Mogabgab and Holmes(11). Approximately 100 $TCID_{50}$ of virus was mixed in equal volume with dilutions of serum, previously inactivated at 56°C for 30 minutes. After remaining at room temperature for 1 hour, the mixtures were inoculated into each of 2 secondary monkey kidney tissue cultures which were incubated in stationary racks at 33°C. The tubes were examined daily for the presence of cytopathic effect. The final readings usually were made 4 to 5 days after inoculation. In all neutralization tests a known guinea pig antiserum control was included and the virus was titrated. Neutralization titers are the reciprocal of the highest dilution which prevented a cytopathic effect of more than 1 or 2 small foci.

TABLE I. Isolation of Viruses from Mild Acute Respiratory Disease.

Week of (1960)	No. of samples		No. viruses isolated	No. identified as ECHO 28
	ARD	Routine		
Oct. 3	17	—	2	1
" 10	10	74	4*	2
" 17	14	—	6	4
" 24	5	—	2	1
" 31	3	—	1	—
Total	49	74	15	8

* Includes 1 unidentified virus from routine sampling.

Preparation of animal antisera. Antisera to JH, 2060 and the prototype virus isolated in this study were prepared in guinea pigs by intramuscular inoculation of 1 ml of undiluted human kidney tissue culture fluid. The inoculations were repeated at weekly intervals over a period of 6 weeks. The animals were bled 10 to 14 days after final inoculation.

Results. The isolation of viruses from samples obtained during a 5-week period beginning Oct. 3, 1960 is shown in Table I. The first routine sampling of the volunteer group as asymptomatic controls was scheduled for the second week of this period. All of the students reported except for 17 who had been sampled during illness in the previous week. During the week of routine sampling 10 students reported with acute respiratory infections and 74 were well. In the following 3 weeks, 22 of the latter reported with respiratory infections. Most of these illnesses were mild, with symptoms of coryza, nasal congestion and sore throat persisting for about one week. However, one student was subsequently hospitalized with a diagnosis of streptococcal pneumonia and 2 others developed infectious mononucleosis.

Fifteen viruses were isolated, 9 in both human and monkey kidney cultures, 6 in human kidney cultures only and none in H.Ep. 2 cell cultures. Eight of the 15 were identified as ECHO 28 by neutralization tests with

TABLE II. Neutralizing Antibody Titers to 3 Strains of ECHO 28 Virus in Paired Sera of Students from Whom This Virus Was Isolated.

Student No.	Neutralization titers: 3/Chicago/60 Acute	3/Chicago/60 Conv.	JH Acute	JH Conv.	2060 Acute	2060 Conv.
118	8	64	8	64	8	128
120	<8	8	<8	8	<8	8
124	<8	>64	16	>64	8	32
150	16	>128	16	>128	16	>128
140	16	>64	8	32	8	32
147	<8	32	8	32	16	32
142	<8	32	8	32	8	32
149	8	>64	16	64	16	64

JH guinea pig antiserum.† One of these strains was selected as a prototype and designated 3/Chicago/60. Neutralizing antibody titers were determined with this virus as well as JH and 2060 on the acute and convalescent sera from the 49 students with acute respiratory infections. Antibody rises occurred only in sera of persons from whom ECHO 28 virus was isolated. Seven of the 8 showed 4-fold or greater increases in titer (Table II) and titers to the 3 strains were very similar. Cross neutralization tests with guinea pig antisera and the 3 strains of ECHO 28 (Table III) failed to reveal significant antigenic differences.

Of 7 viruses listed as unidentified in Table I, one was isolated in both human kidney and monkey kidney cultures. Six grew only in human kidney cultures. One of the latter was isolated from an asymptomatic student during the week of routine sampling. All grew more slowly than ECHO 28, although the cytopathic effect was similar. These viruses were not neutralized by guinea pig antisera to ECHO 28 or to HGP(13) and are still under investigation.

Discussion. Isolation of 8 strains of ECHO 28 virus and demonstration of significant increases in antibody in 7 of the 8 persons from whom the viruses were isolated implicates this agent in mild acute respiratory infections in young adults. Since serological tests failed to reveal any further cases of infection by ECHO 28 it appears that the method used for isolating this virus was quite efficient. Previous reports have established the importance of incubating cultures on roller drums in a medium with a low bicarbonate concentration (5,6,7,12). Our results indicate that a lower temperature of incubation (33°C) as used by Tyrrell *et al.*(13) for HGP virus is also suitable for isolation of ECHO 28. This virus also grew well in secondary human kidney cultures.

All serologic data presented here indicate that the 3 strains of ECHO 28 virus; JH, 2060, and the newly isolated prototype 3/Chicago/60, are antigenically very similar. Although tests with hyperimmune animal sera might not be expected to reveal small antigenic differences, tests with convalescent serum from infected humans should show a higher homologous titer. This was not observed.

The isolation of 7 unidentified viruses suggests a multiple etiology of mild acute respiratory infections during this period.

Summary. Fourteen viruses were isolated from 49 volunteers presenting with symptoms of mild acute respiratory disease during a 5-week period. A 28.6% isolation rate was thus obtained. Samples from 74 asymptomatic students obtained during this period yielded only one still unidentified virus. Eight viruses were identified as ECHO 28. Paired sera of 7 of the 8 subjects from whom this virus was isolated showed a significant increase in neutralizing antibody titer. None of the other subjects with acute respiratory infections demonstrated this antibody response. No antigenic differences among the ECHO 28 strains were detected by neutralization tests employing the newly isolated prototype strain 3/Chicago/60, JH or 2060 with human acute and convalescent sera or with hyperimmune guinea pig sera. Other viruses, as yet unidentified, were also isolated

TABLE III. Cross Neutralization Tests with 3 Strains of ECHO 28 and Their Respective Guinea Pig Antisera.

Guinea pig antisera	Neutralization titers: 3/Chicago/60	JH	2060
3/Chicago/60	*512*	256	256
JH	512	*256*	512
2060	32	32	*64*

† We are indebted to Dr. J. C. Holper of Abbott Laboratories for the antiserum.

at the time ECHO 28 was prevalent.

The authors gratefully acknowledge the technical assistance of Mr. Richard C. Ewert, Miss Mabel Smith and Mrs. Leautry Davis.

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Separation of Brain Proteins by Starch Gel Electrophoresis.* (26750)

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The complex nature of the mixture of proteins occurring in most tissues has rendered their identification and characterization exceedingly difficult. Analysis by conventional Tiselius technics and paper electrophoresis procedures have not yielded definitive results. The use of agar gel and starch gel (1.2) as supporting media for electrophoresis studies provides some superiority over paper in that more fractions have been visualized, and both have been successfully employed for studies of serum proteins. However, the applicability of these procedures to the study of tissue proteins has been limited. Voskobolnokov(3) reported on the separation of liver proteins using agar gel and was able to identify 13 separate moieties. Karcher, van Sande and Lowenthal(4) have reported on the separation of central nervous system proteins on agar gel in various pathological conditions. They characterized the proteins in a manner analagous to serum proteins and demonstrated 7 protein bands in normal white matter and 8 in normal brain stem, using a densitometer to quantitate their material. Eariler studies using paper electrophoresis(5) had inadequately resolved brain proteins although Palladin and Poliakova(6) reported the presence of 7-8 fractions in the central nervous system of the cat using this technic.

In this study, starch gel was used as the supporting medium, and the vertical electrophoresis procedure(7) was adopted. This yielded a satisfactory separation of the protein moiety, which appeared superior to that reported by the other methods mentioned. While this work was in progress Bailey and Heald(8) reported on use of horizontal starch-gel electrophoresis for separation of central nervous system proteins. They achieved some satisfactory delineation of the proteins.

Methods. Adult rats were sacrificed by fracturing the spinal column with the aid of a heavy metal bar, and the brain was extir-

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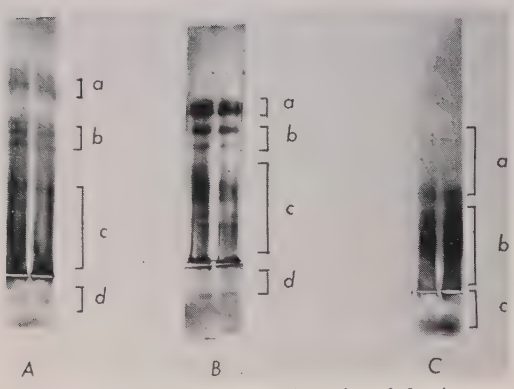

FIG. 1. Starch-gel electrophoresis of brain proteins. A. Rat brain, unperfused. B. Rat brain, perfused. C. Human brain, white matter.

pated immediately. This procedure was performed usually in less than one minute. The brain was placed into one volume (g/ml) of either cold distilled H_2O or isotonic NaCl and homogenized. It was found that both extractives yielded similar results while butanol extraction gave inferior separations. The homogenates were frozen and thawed 5 times and then centrifuged at 2°-4°C at 20,000 × g for 60 minutes. Centrifugation at lower speeds (*viz.* 3500 × g) yielded preparations which gave poor results. Fifty lambda of the homogenate was then placed in the slit of the block. Rat blood was obtained by exsanguination on a different group of animals and the serum run directly.

Human material was obtained from postmortem specimens, usually within 6 hours after death and preparation of the extract was the same as described above.

The starch was obtained from Connaught Medical Research Laboratories, and preparation of the gel as well as separation were carried out as described by Smithies(7).

After some trials a .025M borate buffer at a pH 8.9 was employed for preparation of the starch gel and a voltage gradient of 4.5 volts/cm was applied across the cell. The sample was placed in the slit, then the block was covered with saran wrap after the slit had been overlaid with a mixture of vaseline and mineral oil. The apparatus was then placed in a 13°C incubator and run for 14-18 hours. After fractionation was completed, the starch block was placed on the table and cut in half in a horizontal plane and protein was visualized by staining with Amido black B and washed as described by Pert *et al.*(8).

Results. Rat brain. Separations were performed on white and grey matter and corpus striatum, but because no qualitative differences could be discerned between the homogenates of the 3 structures, the results obtained on whole brain are shown in Fig. 1A & 1B as representative of the type of fractionation achieved. Perfused and unperfused brain are depicted in the unretouched photograph. The two types of preparation have in common the appearance of 2 leading bands, of albumin-like migration characteristics (a). This was demonstrated by running serum albumin simultaneously, and noting that its mobility corresponded to that of the slower of the 2 bands. Both perfused and unperfused brain have in common the appearance of 2 bands migrating toward the cathode (d), presumably γ-globulin-type proteins, and both preparations contain 4-5 bands on the anode side nearest the origin (c). The effect of perfusing the brain is shown in what is presumably the alpha-globulin region where 3 close-appearing bands are seen in the unperfused preparation, but one of these bands is absent in the perfused tissue (b). Whether this represents hemoglobin or is related to the perfusion technic is undetermined. Thus 11-13 bands can be separated and visualized in rat brain by this procedure.

Human white matter. The fractionation achieved with human white matter is shown in Fig. 1C. There are 3 bands migrating toward the cathode (c), then 7-8 light-staining bands appearing in a position analagous to serum albumin and alpha-globulin (a), and 4 bands, characterized by a heavy staining band at about the center of the electrophorogram (b). Whether this represents blood contamination is undetermined. In all, the white matter seems to separate into 14-15 bands compared with the 11-12 obtained above in rat brain. It is difficult to ascertain what contribution blood may make to the proteins visualized since perfusion of this material was not attempted.

Discussion. It is apparent that human white matter contains only a small percent-

age of proteins of albumin characteristics in terms of mobility, and differs markedly from blood serum in this respect. This paucity of albumin in white matter has also been reported in paper electrophoresis studies(9,10), but the results with agar(4) demonstrated appreciable amounts of this type of protein. That these observed differences can be attributed to the two types of technics employed seems improbable, and our data tend to support the results obtained on paper. The rat brain shows considerably more albumin-type protein, as well as a definite specie difference in the number as well as mobility of other protein fractions. Results obtained by other workers with various species would indicate that considerable variations in type and concentration of various brain proteins exist between species. While this might be expected on the basis of an analogy with blood serum, it would seem that less variation might occur in brain, because of the basic neural function of the organ, and its relative independence of normal external conditions to maintain its structural integrity. Whether these differences may ultimately be correlated with some of the higher cerebral function of human brain is a moot point.

No attempt was made to quantitate the data as it was felt that conventional dissection and elution technics presented a problem which is not now too readily soluble. First, the thickness of the gel cannot be controlled during the cutting procedure so that assay of the protein by measurement of 280 mμ absorbing material did not seem feasible, and secondly, determination of dye intensity by extraction of the protein from the starch did not seem satisfactory because of the varying degrees of background staining along the gel. Measurement of color intensity either by a densitometer or reflectometer has not been attempted as it was felt that some of the same criticisms applied. It would seem that characterization of the proteins in brain would be of greater significance than quantitation. Our efforts are being directed to the former problem.

Summary. Starch-gel electrophoresis has been employed to separate the proteins in human and rat brain. This technic yielded results superior to those attained by paper or agar-gel electrophoresis. Separation of water-soluble proteins on starch-gel yielded 11-12 discrete protein bands in rat brain and 14-15 bands in human white matter. Human white matter proteins are characterized by low concentration of albumin-type proteins.

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Production of Neuraminidase by L Forms of *Vibrio cholerae*. (26751)

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Receptor-destroying enzyme (RDE) catalyzes the release of various neuraminic acids from glycoproteins and is therefore a neuraminidase(1). Filtrates with this activity, or the related property of inhibition of hemagglutination by myxoviruses, have been obtained from cultures of *Vibrio cholerae*(2), *Diplococcus pneumoniae*(3), *Clostridium perfringens*(4), and other bacteria(5). An enzyme with similar activity is an integral part of the influenza virus particle(6).

The neuraminidases of *V. cholerae, D. pneumoniae,* and influenza PR-8 virus, although possessing similar catalytic specificity, are serologically distinct(7). The present paper reports that the L form of *Vibrio cholerae* also produces a neuraminidase, which is inhibited by antiserum to the enzyme of the parent strain.

Materials and methods. Influenza virus PR-8 strain in the form of infected chorioallantoic fluid was used. The preparation of *V. cholerae* (strain 4 Z) and *D. pneumoniae* (type I-S) neuraminidases, and of antisera to these enzymes and to the influenza virus, has been described previously(7).

L forms of the *Vibrio cholerae* were produced by the method of Dienes(8). Fresh cultures were plated on nutrient agar containing 10% horse serum and penicillin (1000 units per ml). The L forms obtained by this method were transferred on the same type of agar at weekly intervals for 4 months, then induced to grow in broth by layering 10% horse serum-trypticase-soy broth (Baltimore Biological Laboratories) over agar block cultures in Erlenmeyer flasks. The cultures were incubated at 37°C for 72 hours, with periodic shaking. L forms were identified by the method of Dienes(8). Attempts to induce reconversion to the original bacterial form were unsuccesful, indicating that the L forms were stable. The broth filtrate was centrifuged at 25,000 G for 2 hours in the cold and the supernatant fluid studied for neuraminidase activity.

Virus, neuraminidase and anti-neuraminidase titrations were performed by the hemagglutination methods previously described(7). Specific neuraminidase activity was examined by incubation of the Vibrio L filtrate with highly purified α-1 glycoprotein* at 37°C for one hour and determination of free N-acetylneuraminic acid by the method of Warren (9). Total neuraminic acid was determined using the resorcinol method(10).

Results. Agglutination of chicken erythrocytes by influenza PR-8 virus was inhibited to a 1:32 dilution by Vibrio L broth filtrate. Eight-fold concentration of the filtrate by pressure ultrafiltration(7) resulted in inhibitory titers of 1:128-1:256.

Concentrated Vibrio L filtrate was dialyzed for 48 hours against 0.05M NaCl and titered for inhibitory activity in .155M saline-cacodylate buffer pH 6.8. The hemagglutination titer was reduced to 1:4. Titration of this material in calcium-borate buffer (11) gave titers of 1:64-1:128, indicating the need of the L filtrate enzyme for calcium ions, similar to that of the enzyme of the parent organism. Similarly, addition of 5% sodium citrate or 0.2M disodium ethylenediaminetetracetic acid resulted in sharp disappearance of inhibitory activity.

Antiserum to the neuraminidase of the parent strain of *V. cholerae,* neutralized hemagglutination-inhibition by Vibrio L neuraminidase to nearly identical levels, 1:128-1:256, when titrated simultaneously against 5 hemagglutination-inhibition units of the parent and L form neuraminidase. Antisera to pneumococcal neuraminidase and to influenza PR-8 virus exhibited no inhibitory effect. No attempt was made to produce antiserum to the neuraminidase of the L form.

* Kindly supplied by Dr. Edwin H. Eylar, Harvard Medical School.

Neuraminic acid was released quantitatively from α-1 glycoprotein by incubation with Vibrio L form neuraminidase. Reaction mixtures containing 1000 μg of α-1 glycoprotein yielded 104 μg of neuraminic acid when determined by the resorcinol method and 114 μg when aliquots were measured by the Warren reaction after enzyme action. These results are in close agreement with the expected value of 110 μg, since N-acetylneuraminic acid constitutes 11% of the α-1 glycoprotein. Neuraminic acid was not detected in the Vibrio L filtrate by the resorcinol method.

Discussion. The retention of various properties of the parent strain by L forms of bacteria has been noted previously. Scheibel and Assandri(12) were able to isolate L forms of *Cl. tetani* toxigenic for mice, similar to the findings of Tulasne and Lavillaureix (13) who studied a water-vibrio EZ-5. Kandler and Kandler(14) demonstrated retention of various biochemical functions by L forms of *B. proteus* and *V. cholerae,* although specific enzymatic activities were not investigated.

In the present study, the neuraminidase of *Vibrio cholerae* L form was found to be identical in all respects with the same enzyme of the parent strain. Inhibition of hemagglutination by influenza virus was indistinguishable from that produced by the parent strain and specific release of neuraminic acid from a suitable substrate was easily demonstrable. The neuraminidases of both the parent and L form exhibited the same dependence upon calcium ions for activation. Further evidence of the identical nature of the 2 enzymes is provided by the neutralization studies. Specific antiserum to neuraminidase of the parent strain suppressed the activity of the parent and L form enzymes to exactly the same degree. Absorption studies were not done. It is unlikely that they would have yielded additional information. Scheibel and Assandri(12) noted neutralization of the toxin of *Cl. tetani* L forms by tetanus antitoxin.

Summary. A stable L form of *Vibrio cholerae* (strain 4Z) has been found to produce a neuraminidase capable of inhibiting hemagglutination by influenza virus and of liberating neuraminic acid from purified α-1 glycoprotein. The enzyme activity was neutralized by antiserum to the neuraminidase of the parent *Vibrio cholerae* strain.

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Reversal of Experimental Endotoxin Shock with a Combination of Aldosterone and Metaraminol. (26752)

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A combination of hydrocortisone and metaraminol reversed endotoxin shock in the dog, which was not accomplished with either drug alone(1,2). This report is concerned with the remarkable activity of aldosterone and metaraminol under similar experimental conditions.

Materials and methods. After equilibra-

tion, anesthetized adult mongrel dogs were given a standardized dose of *E. coli* endotoxin; the systemic arterial pressure was monitored over a period of 7 hours, or until death occurred; and arterial pH determinations and hematocrit determinations were done hourly(1,2).

Ten control animals were given endotoxin alone as a test of endotoxin lethality. A second group of 10 animals each received a dose of 2 mg of synthetic d-aldosterone,* and a third group, also of 10 animals, was given 0.2 mg. The steroid was administered intravenously in a solution of 0.1 mg/cc at that time when irreversibility appeared imminent. This stage of canine endotoxin shock is characterized by progressive hypotension, hemoconcentration, acidosis and oliguria or anuria. A fourth group of 25 dogs was treated with a combination of aldosterone and metaraminol.† At the post-endotoxin stage of shock described above, 0.1 mg of aldosterone was injected into the femoral vein of the dog, followed by an infusion of metaraminol. The pressor drug was diluted in 5% dextrose and water and infused in a concentration of 0.2 mg/cc at a rate of 40-60 drops/minute. Total amounts of metaraminol varied between 0.1 and 5.0 mg. The pressor drug was given intermittently post-aldosterone in amounts sufficient to maintain systemic blood pressure between 90-100 mm Hg, or until the dogs showed hemodynamic stability. Serial serum sodium and potassium determinations were carried out in dogs receiving aldosterone alone, using the Baird flame photometer.

Results. Control animals. A dose of 0.55 mg/kg of endotoxin proved lethal in all 10 animals, death occurring in an average of 12.5 hours post-endotoxin.

Aldosterone animals. Two of 10 animals given 0.2 mg of aldosterone survived. Average survival time of the remaining 8 dogs was 8 hours. There were no significant alterations in concentrations of serum sodium and potassium for 7 hours post-endotoxin.

Two of 10 animals receiving the larger dose of 2 mg of aldosterone survived a lethal dose of endotoxin. Average post-endotoxin survival time of animals dying was 8.3 hours. There was no essential change in concentrations of serum sodium in this group; however, the animals exhibited increasing levels of potassium during the observation period. The average increase was from a control of 4.4 mEq/L to a high of 6.5 mEq/L at 4 hours post-endotoxin, in contrast to a maximum increase of 0.4 mEq/L in those animals given the lower dose of aldosterone.

These results show that survival rate of dogs with endotoxin shock was only slightly increased when aldosterone was used alone. The results are comparable to those obtained with hydrocortisone in previous studies(1).

Aldosterone + metaraminol animals. Since dogs appeared to tolerate 0.2 mg of aldosterone without ill effects, and since this dose did not significantly alter the concentrations of serum sodium and potassium, one-half this amount, or 0.1 mg, was infused into 25 animals during the post-endotoxin period of shock, followed by an injection of an amount of metaraminol sufficient to sustain the systemic blood pressure. Sixteen of the 25 dogs survived. Average survival time of the dogs dying was 8.6 hours. Although surviving animals exhibited oliguria and some degree of hemoconcentration, only a minimal degree of acidosis was detected, as determined by blood pH. The average total amount of metaraminol necessary to maintain the systemic pressure was 0.8 mg, the doses varying between 0.1 and 5 mg as compared with an average dose of 42 mg required to maintain the pressure of dogs when metaraminol was used alone in previous studies(1). When hydrocortisone preceded the infusion of metaraminol, 5.5 mg of the pressor drug was necessary.

The over-all results in the 55 dogs receiving 0.55 mg/kg of endotoxin are shown in Table I.

Discussion. Extensive studies on canine endotoxin shock have demonstrated that the reversal of shock offers a more severe therapeutic challenge than either pre-treatment, or simultaneous administration of agents with

* Supplied by Research Dept., Ciba Pharmaceutical Products, Inc., Summit, N. J.

† Supplied as Aramine Bitartrate 10% by Research Division, Merck Sharp and Dohme, Philadelphia, Pa.

TABLE I. Results in 55 Dogs Given a Lethal Dose of *E. coli* Endotoxin.

No. of dogs	Treatment	Survivors	Died
10	None	0	10
10	.2 mg aldosterone	2	8
10	2.0 mg aldosterone	2	8
25	.1 mg aldosterone + avg of .8 mg metaraminol	16	9

endotoxin. This viewpoint has also been expressed by Weil and Miller(3). In this laboratory reversal of shock has been most successful when a combination of adrenocorticosteroid and a pressor drug was used. It has been essential to infuse the steroid before injecting the pressor drug.

The rise in serum potassium observed with the larger doses of aldosterone may be related to the severe oliguria, or anuria, and acidosis that characterize canine endotoxin shock.

We have shown that either glucocorticoid or mineralocorticoid supplements the pressor action of metaraminol. The steroids have 2 favorable effects when used with the pressor drug. First, the amount of pressor agent necessary to sustain the systemic pressure is considerably less when preceded by administration of steroid, especially when aldosterone is employed. Second, although metaraminol will sustain the pressure when used alone, there is no significant increase in survival rate when compared to controls. Steroids not only augment the activity of pressor drug, but rate of survival is remarkably increased. The precise role shared by exogenous steroid is not known. The observations pertain only to endotoxin shock in the dog.

Summary. Lethal shock was established in 55 adult mongrel dogs with a standardized dose of *E. coli* endotoxin. The shock could not be reversed with aldosterone or metaraminol when either was used alone. However, dogs given 0.1 mg of aldosterone required approximately one-fiftieth the dose of metaraminol necessary to maintain systemic pressure, compared to the use of metaraminol alone. This action of aldosterone was about 7 times more pronounced than that obtained with hydrocortisone. The nature of the contributing effect of steroid to the pressor activity of metaraminol and to the increased survival rate is not known.

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Hemorrhagic Reactions at Sites of Passive Cutaneous Anaphylaxis in Guinea Pig after Intravenous Inoculation of Unrelated Immune Complex. (26753)

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A clear cut differentiation between anaphylactic and Arthus-type sensitivities has been afforded by the quantitative studies of Benacerraf and Kabat(1) in the guinea pig. The distinguishing features of transferred Arthus reactions are: (a) The lack of a latency period, the severity of the Arthus reaction being the same when antigen is injected immediately, 30 minutes or 24 hours after the intravenous sensitizing dose of antibody. (b) The requirement of greater amounts of antibody, *viz.*, 400 μg AbN intravenously for severe direct Arthus reactions, as contrasted with 30 μg, *i.e.*, 13 times less, the amount sufficient to convey systemic anaphylactic sensitization. In the case of passive reversed Arthus and of the local anaphylactic reaction known as "Passive Cu-

taneous Anaphylaxis" (PCA), in which antibody is injected intradermally and antigen is given intravenously, the amounts of antibody required for minimal reactions are, respectively, 10 and 0.003 μg AbN(1,2). (c) Non-precipitating rabbit antibody gives only weak Arthus responses, while being equally effective as precipitating antibody in transferring anaphylactic sensitization. (d) On the other hand, horse antibody, while able to elicit strong Arthus reactions when injected intracutaneously in the doses of 36-72 μg AbN, was found entirely ineffective in producing PCA in the guinea pig in the same doses(3).

This body of evidence led Benacerraf and Kabat(1), as well as Ovary and Bier(3) to conclude that Arthus and anaphylactic sensitivities had fundamentally different mechanisms and did not differ only in degree. While this conclusion is probably correct, previous work(4), as well as present experiments strongly indicate that concomitant PCA plays an important role in development of the Arthus reaction.

Materials and methods. 1. *Antisera.* The following antisera were employed: a) *Rabbit anti-bovine serum albumin* (anti-BSA), pools 4, 5, and 6, containing respectively 0.640, 0.560, and 0.405 mg AbN/ml. b) *Rabbit anti-egg albumin* (anti-Ea), pools 10, 11, 20 and 22, containing 0.800, 0.400, 0.450 and 0.650 mg AbN/ml respectively. c) *Rabbit (type III) anti-pneumococcal serum* (anti-SIII), which contained 0.700 mg AbN/ml. 2. *Antigens. Bovine serum albumin* (BSA), crystallized, obtained from Pentex, Kankakee, Ill.; *Chicken egg albumin* (Ea), prepared by the method of Kekwick and Cannan(5); and *SIII*, lot 202, a purified preparation kindly supplied by Dr. M. Heidelberger. 3. *Complement titrations.* Complement (C′) titers were estimated according to the technic described in(6) in serum samples obtained from blood (1-2 ml) drawn by cardiac puncture. C′3 titers were determined with R3, as described in(7). 4. *Complement fixation.* Complement-fixation titers were expressed in terms of the amount of AbN required for 50% lysis in the presence of 5 units of C′. 5. *Preparation of immune-complexes.* Immune-complexes of different composition were prepared from rabbit serum anti-BSA and crystallized BSA. Immune precipitates (IP) 1, 2, and 3 were obtained by mixing 2 ml of antiserum with amounts of antigen corresponding to 0.2, 0.5 and 1.0 the equivalent dose. Mixtures were allowed to stand overnight at 0°C and precipitates were washed 3 times with chilled saline. The amount of AbN in the precipitates was then determined by use of biuret reagent.

For preparation of soluble immune-complexes (SC) 4, 5, 6, and 7, washed precipitates obtained at the equivalence zone, as described above, were resuspended in 2 ml of saline with an excess antigen corresponding to 2, 5, 10, and 40 times the equivalent dose. Mixtures corresponding to 2 and 5 times excess antigen did not dissolve completely after 24 hour incubation in the ice box. The AbN content of their precipitates was determined under the assumption that the ratio A/G was the same as in the original precipitate and the AbN content of corresponding supernatants (SC 1 and SC 2) was estimated by difference from total antibody initially present. Precipitates resuspended in 10 and 40 times excess antigen were completely dissolved so that the AbN/ml of their supernates (SC 6 and SC 7) could be estimated on the basis of antibody content of the original precipitates. The AbN content per ml of the immune-complexes was the following: IP 1 – 0.354 mg; IP 2 – 0.570 mg; IP 3 – 0.540 mg; SC 4 – 0.110 mg; SC 5 – 0.330 mg; SC 6 and SC 7 – 0.540 mg. The IP suspensions, as well as the SC supernatants were diluted to yield 0.100 mg AbN/ml and further diluted as required for the experiments. 6. *Animals and technic of injections.* Guinea pigs of 250 ± 50 g were used throughout. Intracutaneous injections were made into the skin of previously clipped areas of the belly with a short-beveled 26 gauge needle, first introduced through the skin, then reversed in direction so as to deposit the inoculum, 0.1 ml, into the dermal layers. Intravenous injections were made also with 27 gauge needles into superficial veins of hind foot or of ear.

Results. Effect of simultaneous PCA on passive direct Arthus reaction. To investi-

TABLE I. Effect of Simultaneous PCA on Passive Direct Arthus Reactions Induced in Guinea Pigs by Different Amounts of Antibody from an Unrelated Immune System.

Anti-EaN* i.v.	Virgin site† (0.05 mg EaN)				PCA site† (Anti-BSA)				PCA site† (Anti-SIII)			
μg AbN					1.5 μg AbN				1 μg AbN			
400	+	2+	3+	4+	3+	3+	3+	2+				
	±	±	4+	4+					3+	0	+	2+
200	0	0	+	2+	3+	3+	±	±				
	0	±	±	+					3+	0	3+	3+
100	0	0	0	0	+	2+	3+	4+				
	±	0	0	0					3+	+	3+	0
50	0	0	0	0	+	2+	3+	3+				
	0	0	0	0					2+	+	2+	3+
25	0	0	0	0	+	2+	2+	3+				
	0	0	0	0					3+	3+	3+	0
12	0	0	0	0	±	+	3+	4+				
	0	0	0	0					0	4+	+	2+
6	0	0	0	0	+	+	2+	3+				
	0	0	0	0					+	+	+	±
3	0	0	0	0					±	0	+	+
0	0	0	0	0	0	0	0	0				
0	0	0	0	0					0	0	±	±

* Mixed with 0.2 mg BSA or 0.1 mg SIII.

† All reactions graded are of the Arthus type. Reactions recorded in horizontal rows in corresponding vertical columns, at virgin and PCA sites, were produced in the same animal (see text).

gate whether concomitant PCA had any effect on direct Arthus reactions elicited by unrelated immune system, guinea pigs were injected intradermally (PCA site) with 1.5 μg anti-BSA N (or 1.0 μg anti-SIII N) and 1 hour later received Ea intracutaneously at a virgin site and a mixture of anti-Ea + BSA (or SIII) by the intravenous route. Animals were killed 2 hours after challenge and the diameter of reactions measured on the internal side of skin. Intensity of reactions was graded as 4+ (more than 20 mm diameter), 3+ (15-20 mm), 2+ (10-15 mm) + (5-10 mm), ± (less than 5 mm), and 0 (no reaction). The data summarized in Table I show that severe hemorrhagic reactions developed at site of injection of Ea (virgin site) only in the animals sensitized with 0.400 mg anti-EaN. Strong reactions appeared, however, at the PCA site (anti-BSA or anti-SIII) with amounts of the Arthus-producing antibody as little as 0.012 or even 0.006 mg AbN.

Development of hemorrhagic reactions at PCA sites after i.v. *inoculation of unrelated immune complex.* The foregoing experiment suggests that Arthus-producing antigen injected into virgin site was partly absorbed and formed complexes with its corresponding antibody in circulating blood, which selectively localized at PCA site. Following experiments were performed to check this interpretation. In a first series of experiments (Table II), different amounts of a soluble immune complex prepared with Ea/anti-Ea were mixed with BSA and injected intravenously into animals previously sensitized with anti-BSA by the intradermal route. Vice versa, BSA/anti-BSA was mixed with Ea and injected intravenously in guinea pigs previously sensitized with anti-Ea.

In a second series of experiments (Table III), BSA/anti-BSA complexes of different composition, corresponding to excess antibody, equivalence, and excess antigen, were mixed with Ea and injected intravenously in animals previously sensitized with anti-Ea. In both series of experiments, hemorrhagic reactions (2 or 3+) developed at PCA sites with amounts of intravenously injected complex corresponding to relatively low levels of antibody (*cf.* Tables II and III).

Parallelism between hemorrhagic reaction,

TABLE II. Hemorrhagic Reactions Induced by Soluble Complexes of Ea/Anti-Ea at PCA Sites from BSA/Anti-BSA and *Vice versa.*

AbN/ml of immune complex	Hemorrhagic reactions at PCA sites: Anti-BSA						Anti-Ea					
Ea/anti-Ea												
100	4+	3+	0	2+	+	3+						
25	+	2+	2+	3+								
12	+	2+	3+	3+								
6	3+	+	2+	0								
BSA/anti-BSA												
100							3+	3+	+	0	2+	3+
25							2+	±	4+	3+		
6							3+	±	±	+	+	3+

1 ml of soluble complex inj. i.v. together with PCA antigen 2 hr after intradermal inj. of 1 μg AbN of PCA antiserum. Each reaction corresponds to a separate animal.

TABLE III. Hemorrhagic Reactions Induced at PCA Sites by Intravenously Injected Immune-Complexes of Different Composition.

Immune-complex	Amt of antigen in relation to equivalent dose	AbN/ml of immune complex, μg: 100	25	6.25	μg AbN for fixation of 4 from 5 $C'H_{50}$
IP 1	1/5	2+	3+	±	.22
		2+	2+	0	
IP 2	1/2	+	+	0	.18
		3+	3+	+	
IP 3	1	+	+	+	.18
		+	+	+	
SC 4	2	2+	2+	+	.66
		3+	2+	+	
SC 5	5	2+	±	0	.87
		+	±	±	
SC 6	10	2+	0	0	
		+	2+	0	
SC 7	40	0	2+	±	.84
		+	±	0	

1 ml of immune-complex inj. intrav. together with 0.1 mg EaN 2 hr after intradermal inj. of 1 μg anti-EaN. Each reaction corresponds to a separate animal.

carbon granulopexis and dye accumulation. Two different phenomena occur at PCA sites —the diffusion of intravenously injected dye as a consequence of increased permeability of minute vessels and increased accumulation of colloidal particles (granulopexis) resulting from stimulation of endothelial cells by local liberation of histamine(8).

It was deemed of interest to investigate whether by using 3 different indicators, *viz.*, immune complex, Geigy Blue or colloidal carbon,* parallel results would be obtained. This is clearly shown in Table IV, either in relation to the dose of PCA-eliciting antibody, or in regard to time after challenge with PCA antigen.

Discussion. The data presented here show that severe hemorrhagic reactions (2 to 3+) of the Arthus type develop in the guinea pig skin at PCA sites induced by an unrelated immune system with amounts of intravenously injected antibody as low as 6 μg AbN (Table I). The occurrence of severe Arthus reactions after intravenous sensitization with large amounts of antibody followed by intradermal injection of antigen(1) may be associated to the rapid anaphylactic sensitization obtained with such amounts as 0.400 mg AbN. According to Ovary and Osler(8) the latent period for whole skin sensitization after intravenous inoculation of 0.500 mg AbN is reduced to 1 hour, so that the 2 hour period allowed for development of Arthus reaction in the guinea pig would be sufficient to include participation of a PCA reaction at the site of intradermal injection of Arthus-eliciting antigen. As the amount of intravenously injected antibody is decreased, the latent period for general sensitization becomes progressively greater, *e.g.*, 4 hours for a dose of 0.250 mg AbN(9). By this time the intradermally injected antigen may have been absorbed from the skin depot and formed immune complexes, which are quickly cleared from the blood stream. These complexes may, however, localize at PCA sites

* India ink. Lot C11/1431a, with particles of approximately 250 Å in diameter, obtained from Günther-Wagner, Hannover, West Germany.

TABLE IV. Parallelism between Dye Accumulation, Carbon Granulopexis and Hemorrhagic Reaction Induced by Soluble Immune Complex at PCA Sites in Guinea Pigs.

(A) In relation to dose of PCA antibody

Anti-BSA N intradermally, μg*	Hemorrhagic reactions by immune complex				Dye accumulation				Carbon granulopexis			
1.0	0	2+	3+	3+	4+	4+	4+	4+	3+	3+	4+	4+
.2	±	+	+	2+	2+	3+	3+	4+	2+	2+	4+	4+
.05	0	0	0	2+	0	2+	2+	3+	0	+	2+	2+
.01	0	0	0	0	0	0	0	0	0	0	0	+

* .2 mg BSA i.v. 2 hr later together with .5 ml .5% Geigy Blue, 8 mg colloidal carbon/100 g body wt or 400 μg EaN/100 μg anti-EaN. Reactions read 2 hr later. Each vertical line corresponds to an individual animal.

(B) In relation to time after inj. of PCA antigen

Min. between i.v. injections†	Hemorrhagic reactions by immune complex							Dye accumulation							Carbon granulopexis						
0	±	±	2+	3+	4+	4+		4+	4+	4+	4+				3+	4+	4+	4+			
15	±	±	±	+	+	2+	2+	2+	2+	3+	3+	3+	3+	3+	±	2+	2+	2+	2+	2+	2+
30	±	±	+	+	+			0	0	0	0	0	±	+ +	0	0	0	0	0	0	
60	0	0	0	±	±	±		0	0	0					0	0	0				

† Guinea pigs sensitized with 1 μg anti-BSA N intradermally and inj. i.v., 2 hr later, with 0.2 mg BSA N. Immune complex (Ea/anti-Ea), dye or colloidal carbon, inj. at intervals specified above. Reactions read 2 hr after last intrav. inj. Each reaction corresponds to an individual animal.

and there elicit hemorrhagic reactions (Table I).

The effectiveness of horse antibody in regard to the Arthus reaction is in apparent contradiction to its inability to promote PCA. However, as demonstrated by Osler *et al.*(10), horse anti-pneumococcus serum is only less active than rabbit antiserum both in regard to C′ fixation, and in conferring PCA to the rat. This is in agreement with the observations of Benacerraf and Kabat (1) that dircet Arthus reactions induced by horse antibody in the guinea pig skin are considerably less severe than those caused by equal weight of rabbit antibody.

In the experiments reported by Ovary and Bier(3), in which 36-72 μg AbN corresponding to 0.1-0.2 ml undiluted horse typhoid serum were injected intradermally, challenge with antigen plus Geigy Blue was delayed until 8 hours later to avoid non-specific effect of concentrated serum and negative PCA results were observed. While it is generally admitted that horse antibody does not fix on the guinea pig skin, the possibility can not be excluded that large amounts of horse antibody will induce immediate and transitory PCA sensitivity. However, demonstration of this specific anaphylactic sensitivity is not feasible on technical grounds because of the simultaneous non-specific irritating effect of concentrated or undiluted horse serum.

From the evidence that concomitant PCA enhances the noxious effect of immune complex it does not follow that the local anaphylactic reaction is a necessary prerequisite for development of the Arthus reaction. It is noteworthy, however, that the ability of soluble immune complexes and of aggregated gamma globulin preparations to induce hemorrhagic reactions after single intracutaneous injection in the guinea pig(11) parallels their activities in promoting C′ fixation, as well as in eliciting local accumulation of dye. Experiments are under way on the behavior of aggregated gamma globulin, from various species as to their abilities to induce hemorrhagic reactions at PCA sites when inoculated by the intravenous route.

Summary. Experimental evidence is presented pointing to the prime importance of simultaneous anaphylactic sensitivity in development of hemorrhagic reactions of the Arthus-type in guinea pigs. A final conclusion as to the mechanism by which the local anaphylactic reaction enhances endothelial

damage by intravenously injected immune complex requires further study.

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Flavokinase Activity of Rat Tissues and Masking Effect of Phosphatases.* (26754)

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Riboflavin-5′-phosphate or flavin mononucleotide (FMN) is an essential coenzyme form of the vitamin, riboflavin, and occurs widespread in plant and animal organisms (1). However, scanty information is available on the ability of animal tissues to synthesize FMN from riboflavin. It was suggested that intestinal mucosa is the site of this conversion within the mammal(2) by either a transphosphorylation catalyzed by non-specific alkaline phosphomonoesterase(3) or a phosphorylation requiring adenosine-5′-triphosphate (ATP) and the specific flavokinase(4). With the recent partial purification and characterization of flavokinase from rat liver(5), the ability of a mammalian tissue other than intestine to form the coenzyme *in situ* has been demonstrated. In this work, the recognition of interfering phosphatases which hydrolyze FMN, thereby decreasing formation of FMN with flavokinase *in vitro,* has led to the current assessment of these enzymes in animal tissues. This report demonstrates that several rat tissues possess flavokinase, and the level of apparent activity is markedly suppressed by phosphatases which are maximally active at pH 5.

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Methods. Female, 200 g Sprague-Dawley rats were sacrificed by rapid decapitation and exsanguination. Tissues were excised, rinsed and homogenized in 4 volumes of cold, 0.05 M potassium phosphate buffer, pH 7. Homogenates were centrifuged in the cold at 18,500 × g for 30 min to obtain the supernatant solutions. Protein was determined by the method of Lowry *et al.*(6).

Mixtures for determining flavokinase activity contained: 10^{-4} M riboflavin, 10^{-3} M ATP, 10^{-4} M Zn^{++}, 0.075 M potassium phosphate buffer, pH 8, and 1 mg of protein in 5 ml total volume. Trichloroacetic acid was added to stop the reaction before and after incubation was carried out in the dark at 37°C for 1 hr. The product, FMN, was determined by Kearny's adaptation(7) of the differential extraction method of Burch *et al.* (8). In addition, the product remaining after removal of excess riboflavin with benzyl alcohol was applied to Whatman No. 1 paper and the spots detected under U.V. light following development in ascending *n*-butyl alcohol/acetic acid/water (4/1/5, upper phase). Rf values agreed with those reported

TABLE I. Distribution of Flavokinase and the Masking Effect Due to Phosphatases.

Tissue supernatant	△ mμmoles FMN* Apparent formation: Extraction(8)	Chromatography(9)	Maximum hydrolysis†
Liver	14	++++	88
Kidney	9	++++	90
Brain	4	+++	76
Spleen	2	++	74
Heart	1	+	51
Intestine (small)	<1	—	40

* Values represent avg of 3 to 5 animals with S.D. <25%.

† 10^{-4}M FMN replaced riboflavin and ATP under conditions used for kinase assays with 10^{-4}M Zn^{++} and 0.075 M potassium phosphate buffer, pH 8.

for FMN(9), and no other flavin products could be detected.

In determining phosphatase activity under conditions optimal for flavokinase, incubation mixtures contained 10^{-4} M FMN in place of riboflavin and ATP. Under optimal conditions for the phosphatase, 0.5 mg of protein in 10^{-4} M FMN and 0.075 M potassium acetate buffer, pH 5, was incubated for 30 min. Hydrolysis of FMN was measured by the differential extraction procedure (8) and by liberation of inorganic phosphate using the method of Fiske and Subbarow (10).

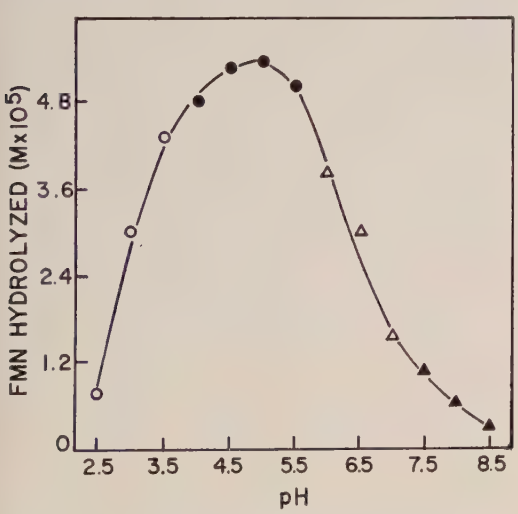

FIG. 1. pH optimum for hydrolysis of FMN by phosphatase from rat liver. 5 ml incubation mixtures contained: 10^{-4}M FMN, 0.5 mg protein, and 0.075 M buffers of glycine-HCl (○), potassium acetate (●), tris-maleate (△), or tris-HCl (▲).

Results. The distribution of flavokinase within rat tissues and the significant masking effect of phosphatases under conditions used to assay the kinase are shown in Table I. Liver appears to contain relatively high kinase activity which is present in decreasing amount in extracts from kidney, brain, spleen, heart, and intestine, respectively. All of the measurable kinase activities may be increased several fold when account is taken of the extensive hydrolysis of FMN which is possible under these assay conditions.

The optimum for hydrolysis of FMN under conditions where rate is approximately zero-order (excess substrate) is near pH 5, as shown in Fig. 1 for the liver phosphatase.

TABLE II. Hydrolysis of FMN by Phosphatases at pH 5.

Tissue supernatant*	mμmoles FMN hydrolyzed†
Kidney	345
Liver	252
Spleen	232
Brain	210
Heart	152
Intestine (small)	135

* Preparations containing 0.5 mg protein were those used in Table I.

† The same relative order of activities was found using whole homogenates.

p-Nitrophenylphosphate behaves similarly as substrate with the same pH optimum and approximately the same rate; however, FMN is much more readily hydrolyzed than adenosine-5′-monophosphate > α- or β-glycerophosphate > fructose-6-phosphate. Other tissues examined (Table II) possess the FMN phosphatase activity in varying amounts with kidney extracts showing the greatest specific activity.

Discussion. The occurrence of flavokinase in several rat tissues, *e.g.*, liver, kidney, brain, spleen, and heart, in addition to its previously reported presence in intestine(4), would support the belief that this enzyme may play an important role in maintaining adequate flavin coenzyme levels in this mammal(5). Intestinal synthesis of FMN is probably not the sole means for supply to the other organs. Moreover, the presence of masking phosphatases may account for

the low activities of kinase measured in extracts from animal tissues. Hydrolysis of FMN by spleen homogenates has been noted previously(11). The physiological significance of the pH 5 phosphatase may be to maintain a check on the intracellular level of FMN. This phosphatase is relatively specific as little activity is found with several organic monophosphates tested as substrates. More extensive examinations of the partially purified enzyme are being made.

Summary. Flavokinase is present in extracts from several rat tissues with activity in liver > kidney > brain > spleen > heart. Phosphatases, maximally active at pH 5, partially mask the kinase activity in these extracts. It is concluded that synthesis of FMN occurs in several organs rather than intestine alone.

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Distribution of Blood Flow to the Canine Heart.* (26755)

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Total blood flow to an organ can be measured by several methods. The distribution of blood or regional flow within an organ is more difficult to quantitate. In the present investigation radioactive microspheres were used to measure regional distribution of blood in the myocardium of the dog and to estimate the size and volume flow through intercoronary anastomoses and arteriovenous shunts.

Methods. The method used is a modification of the technic of Prinzmetal *et al.*(1) developed by Grim and Lindseth(2). In these experiments glass microspheres, 20 μ in diameter, rendered radioactive by the conversion of some of the Na^{23} content of glass to Na^{24} by neutron bombardment, were injected into the coronary arteries of the dog. With the reasonable assumption that the spheres are distributed at arterial bifurcations in the same proportion as is the blood, the fraction of the injected spheres which passed through arteriovenous anastomoses could be quantitated by collecting all venous drainage and counting it in a scintillation counter. Likewise, since those spheres that remained behind in any particular tissue presumably did so because they lodged in vessels smaller than the diameter of the spheres, the "sphere count" recovered from this tissue would provide a measure of flow through vessels smaller than the spheres. If the spheres were small enough to pass through all arteriovenous shunts except capillaries, the radioactivity of the tissue would be a measure of tissue capillary flow. For the purposes of the present study tissue blood flow refers to flow through vessels 20 μ or smaller. This includes all true capillaries (less than 12 μ) and capillary-like vessels(16-20 μ).

The fraction of spheres which entered the myocardium supplied by the anterior de-

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† Markle Scholar in Medical Sciences.

scending branch of the left coronary artery after ligation of this vessel would provide a measure of flow through intercoronary anastomoses at least the size of the spheres used.

Preparation of microspheres. Glass microspheres‡ ranging in size from 5 to 200 μ were fractionated into homogenous samples by multiple elutriations. The spheres were subjected to neutron bombardment in the atomic pile at Oak Ridge National Laboratory. A small group of spheres was suspended in a flask of 1.5% gelatin. The mixture was stirred to keep the spheres in suspension. Ten to 20 μl of the suspension containing between 500-1000 spheres were drawn into a short section of polyethylene tubing and radioactivity within the tubing measured in a scintillation counter both before and after delivery to the dog.

Preparation of animal. Adult, mongrel dogs of either sex, anesthetized with sodium pentobarbital (33 mg/kg) and heparinized with 1.5 mg/kg, were used. The right carotid artery was exposed and cannulated with a polyethylene catheter which was advanced *via* the brachiocephalic artery into the ascending aorta. This catheter was used for coronary artery perfusion and for injection of the radioactive microspheres. Tapes were placed about the superior and inferior vena cavae and brachiocephalic artery. The azygos vein was ligated. The aortic arch was dissected to permit placement of an occluding clamp on the aorta immediately distal to the brachiocephalic artery.

At the beginning of experiment inflow, occlusion was established by drawing tight the tapes about the vena cavae. The tape about the brachiocephalic artery was tightened onto the catheter within its lumen. Perfusion *via* the catheter placed in the ascending aorta was started after 4 or 5 cardiac cycles. Heparinized blood at 38°C was injected from a calibrated siliconized glass cylinder with a pressure of 135 mmHg. Blood was collected from the right ventricle and coronary sinus by inserting a catheter *via* the right atrial appendage into the right ventricle. Holes were present in the catheter so that drainage from both ventricle and atrium was assured. When coronary flow in the beating heart was well established and after the aorta was clamped distal to the brachiocephalic vessels, the radioactive microspheres were injected into the perfusion catheter. Perfusion was continued for several minutes and volume flow was noted. Following this, perfusion was discontinued, all luminal blood was collected, and the heart was removed and weighed. The heart was divided into right ventricle, left ventricle supplied by the anterior descending coronary artery, left ventricle supplied by the circumflex artery and the septum. The ascending aorta, pulmonary artery, venous drainage from the heart and the catheters used were separately labelled for counting. All tissue was divided into 2 g samples and placed into glass vials. Radioactivity of all samples was determined using a well scintillation detector. In some experiments the left anterior descending (L.A.D.) artery was cannulated. Perfusion and sampling was carried out otherwise as described.

The distribution of microspheres throughout the heart was determined. Total flow to the heart was measured from the fall in the calibrated reservoir over a timed period. From these 2 measurements flow in ml/g/min was calculated for specific anatomic regions. Only experiments in which at least 95% of the injected spheres were recovered are reported.

Four types of experiments were performed: (1) Total perfusion of the normal dog heart; spheres 20 μ in diameter—3 dogs. (2) Perfusion of the dog heart with left anterior descending coronary occlusion; spheres 20 μ in diameter—7 dogs. (3) Perfusion of the left anterior descending coronary artery; spheres 20 μ in diameter—7 dogs. (4) Perfusion of the left anterior descending coronary artery; spheres 50 μ in diameter.

Results and discussion. The results are illustrated in Tables I to IV. In the normal dog heart microspheres 20 μ in diameter were distributed uniformly throughout the myocardial mass. Hence, the calculated mean tissue blood flow to the right ventricle was 1.36 ml/g/min, to the distribution of the L.A.D.

‡ Kindly supplied by Dr. John Ryan, Minnesota Mining and Manufacturing Co., St. Paul.

TABLE I. Fractional Distribution of 20 μ Spheres in Normal Dog Heart with Calculated Blood Flows.

Exp. No.	Fraction of spheres recovered R.V.	L.A.D.	S.&C.	R.A.	L.A.	Total flow, ml/min.	A/V shunts	Flow, ml/g/min. R.V.	L.A.D.	S.&C.	R.A.	L.A.
1	.19	.15	.55	.02	.03	83	6.0%	1.4	1.4	1.8	.48	.85
2	.29	.20	.41	.04	.03	60	3.0%	1.7	1.5	1.32	.40	.85
3	.29	.12	.49	.03	.03	103	4.0%	.94	.60	.66	.45	.45
Mean	.26	.16	.48	.03	.03		4 %	1.36	1.17	1.26	.44	.58

R.V., right ventricular myocardium; L.A.D., myocardium supplied by left anterior descending coronary artery; S., septum; C., myocardium supplied by circumflex artery; R.A., right atrium; L.A., left atrium; A/V, arterio-venous.

1.17 ml/g/min and to the septum and circumflex distribution 1.26 ml/g/min. Four percent of total flow passed through arteriovenous shunts 20 μ or greater in diameter. The distribution of spheres was: right ventricle 26%; myocardium supplied by the L.A.D. 16%; the myocardium supplied by the circumflex and the septum 48%; the right and left atria 3% to each.

There was a marked decline in number of spheres which reached the myocardium supplied by the L.A.D. after ligation of this vessel (Table II). Instead of 16% of total spheres injected (tissue flow of 1.17 ml/g/min) reaching this area of myocardium, only 3% of total flow or .29 ml/g/min reached this area. It is assumed that they traversed intercoronary anastomoses 20 μ or greater in size.

Approximately 80% of 20 μ spheres remained in the distribution of the L.A.D. when this vessel was perfused. Twenty percent of the total spheres injected passed *via* communications 20 μ or greater in size: 3.0% *via* arteriovenous shunts to the coronary sinus or lumen of the right ventricle; and *via* arterial collateral circulation 6% to the right ventricle, 8% to the septum, and 3% to the myocardium supplied by the circumflex artery (Table III).

A very similar distribution existed when 50 μ spheres were injected into the L.A.D. artery (Table IV) hence it may be concluded that the interarterial and arteriovenous shunts that exist are greater than 50 μ and that very few communications between 20 and 50 μ exist.

Microspheres were never found in the luminal blood of the left ventricle indicating competence of the aortic valve in Exp. 1 and 2. This finding casts some doubt on the existence in the dog of left arterioluminal shunts. These vessels, described by Wearn (3), are believed capable of shunting blood either into or from the ventricular lumens and thereby provide a potential source of collateral flow to ischemic muscle. Furthermore, all arterioluminal and arteriovenous shunts on the right side do not appear to exceed 5% of total flow.

This method provides a quantitative measurement in the heart of flow *via* arterial collaterals and arteriovenous shunts. The size of the vessels in question can be precisely de-

TABLE II. Total Perfusion Dog Heart with L.A.D. Coronary Artery Occlusion Injection Glass Microspheres 20 μ in Diameter.

Exp. No.	Fraction of spheres recovered R.V.	L.A.D.	S.&C.	R.A.	L.A.	Total flow, ml/min.	A/V shunts	Flow, ml/g/min. R.V.	L.A.D.	S.&C.	R.A.	L.A.
5	.28	.01	.60	.03	.04	73	4.0%	1.20	.03	1.50	.46	.35
6	.21	.02	.58	.08	.07	93	4.0%	.67	.19	.85	.86	.61
7	.28	.01	.60	.06	.04	43	1.0%	.44	.02	.34	.36	.28
8	.25	.08	.56	.03	.05	118	3.0%	2.20	.99	2.38	1.04	1.43
9	.23	.02	.64	.02	.06	72	3.0%	1.50	.23	1.51	.72	1.00
10	.18	.02	.74	.02	.02	108	2.0%	1.55	.30	2.47	.73	.71
11	.18	.05	.69	.01	.05	130	2.0%	.74	.28	1.20	.38	.86
Mean	.23	.03	.63	.04	.05		2.6%	1.18	.29	1.46	.65	.75

TABLE III. Perfusion L.A.D. Coronary Artery Injection Glass Microspheres 20 μ in Diameter.

Exp. No.	Fraction of spheres recovered R.V.	L.A.D.	S.	C.	Total flow, ml/min.	A/V shunts	Flow, ml/g/min. R.V.	L.A.D.	S.	C.
29	.06	.86	.04	.01	60	3.0%	.27	2.60	.24	.07
30	.01	.92	.02	.02	20	3.0%	.01	.72	.03	.04
31	.07	.82	.04	.05	33	2.0%	.26	1.40	.13	.12
32	.02	.83	.08	.03	20	4.0%	.02	.68	.11	.01
33	.05	.66	.21	.04	115	4.0%	.21	1.40	.83	.16
34	.10	.73	.06	.07	48	4.0%	.08	.62	.14	.06
35	.09	.73	.13	.02	65	3.0%	.23	1.56	.06	.02
Mean	.06	.79	.08	.03		3.3%	.17	1.3	.22	.07

TABLE IV. Perfusion L.A.D. Coronary Artery Injection Glass Microspheres 50 μ in Diameter.

Exp. No.	Fraction of spheres recovered R.V.	L.A.D.	S.	C.	Total flow, ml/min.	A/V shunts	Flow, ml/g/min. R.V.	L.A.D.	S.	C.
50	.04	.78	.02	.13	18	3%	.01	.70	.03	.17
51	.06	.78	.03	.07	50	6%	.04	.76	.04	.07
52	.04	.92	.01	.01	15	2%	.04	.67	.15	.15
53	.01	.90	.04	.02	24	3%	.01	.60	.06	.05
Mean	.04	.84	.03	.06		3.5%	.03	.68	.07	.11

termined and the value of operative or other procedures or factors believed to increase intercoronary anastomoses might be more quantitatively assessed.

The beads used in the present study were completely spherical. The small number injected did not change the flow, pressure, resistance relationships that existed in the heart prior to their injection. Preliminary studies revealed that long periods of perfusion after injection of the microspheres did not increase the number of spheres which passed through arteriovenous shunts over that which existed with very short periods of perfusion.

Summary. A known quantity of radioactive glass microspheres either 20 or 50 μ in diameter were injected into the coronary system of the dog heart being perfused at a known and physiological rate with oxygenated blood at 38°C. With the assumption that spheres were distributed at arterial bifurcation in the same proportion as blood, the fraction which appeared in the venous drainage was taken as a measure of arteriovenous shunts the size of or larger than the spheres injected. Those spheres which remained in the tissue provided a measure of flow through vessels smaller than the spheres. Since the spheres were small enough to pass through all arteriovenous shunts except capillaries, the radioactivity of the tissue was a measure of tissue capillary flow (less than 20 μ for the purposes of the present study). There was a uniform distribution of spheres in the normal dog heart. A calculated tissue blood flow of approximately 1.2 cc/g/min existed in the right ventricle and throughout the left ventricle. This flow diminished to approximately .30 cc/g/min in the distribution of the left anterior descending coronary artery after ligation of this vessel. Evidence is presented to suggest that arteriovenous shunts account for 2-4% of total flow across the heart and that these vessels are probably greater than 50 μ in diameter. No evidence for arterioluminal shunts 20 μ or greater in size communicating with the left ventricle was found.

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Replication of Virus Plaques. (26756)

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The replica plating technic devised by Lederberg and Lederberg(1) has been found to be a most useful tool in research dealing with various facets of bacterial genetics. With the progress made in the field of the genetics of animal viruses, stimulated by the advances in tissue culture and plaque technics(2), it seemed that a similar replica plating technic would be useful in animal viral research. This report concerns the development of such a technic.

Materials and methods. Virus and Tissue culture. Both Eastern (EEE) and Venezuelan (VEE) equine encephalomyelitis viruses were used. The former was obtained from the Communicable Disease Center, U. S. Public Health Service. It was a human strain that has been passed in mice and embryonated eggs. The latter was obtained from a donkey that died of encephalitis, and was passed in embryonated eggs. Chick embryo working seeds were prepared by the usual methods(3). Chick embryo fibroblasts were prepared from 10-day-old chick embryos by the trypsinization method of McClain and Hackett(4). They were used in a suspended cell plaque assay method described below.

Plaque assay. The plaque assay method was a modification of the second method described by Cooper(5) which involved infecting cells previously suspended in an agar overlay from an inoculum placed on the hardened agar surface. Five ml of nutrient agar (4), which contained 0.5% gelatin instead of albumin plus 5% calf serum, was poured as a base into 60-mm Petri dishes. An appropriate volume of chick fibroblasts prepared previously in concentrations ranging from 10-25 million per ml were centrifuged (at 1500 rpm for 5 minutes), resuspended, and mixed in a volume of nutrient agar at 43°C to give a concentration of 50 million cells per ml. One and a half ml of this suspension in agar was poured as an overlay into the Petri dishes and allowed to harden. The infection by virus before incubation at 37°C could be carried out with all materials held at room temperature, but a significant and reproducible increase (3×-6×) in sensitivity of the plaque assay by this procedure was achieved as follows: Plates containing cells were refrigerated for 20 minutes before use. Dilutions of virus seed were made in cold beef heart infusion broth. Fifteen-hundredths ml of appropriate dilutions were inoculated onto the cold plates and spread immediately by rotating the plates. The plates were then placed in the refrigerator for 30 minutes, withdrawn, and incubated right side up for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2. Plaques could be seen by oblique light, but better visualization was obtained by adding a 1/10,000 aqueous solution of neutral red to the plates. It is important in this method of plaque assay that all plates be kept on level surfaces during all manipulations to assure an even distribution of plaques.

Replication of plaques. Replication was carried out in a manner analogous to that described by Lederberg and Lederberg(1) for bacterial colonies. A piece of sterile velveteen was tightened securely over an inverted No. 10 rubber stopper by a celluloid ring. The stopper was connected to a brass base by a metal rod (Fig. 1). The master plate, whose plaques were not developed by addition of neutral red, was inverted and lowered on to the velveteen to make the imprint, removed, and the replica plates in turn inverted and lowered to touch the velveteen. Incubation was carried out at 37°C. After replication, the plaques on the master plate were developed and treated with mercuric chloride as described below.

"Fixing" of plaques. Because plaques fade after a few days, it became desirable to find a simple method to "fix" the location of the plaques and at the same time increase the contrast so that they could be counted and/or photographed when convenient(6). It was found that 2-5% solutions of mercuric

FIG. 1. Apparatus used for replication.

chloride poured over the agar and held for one-half hour before being poured off deepened the red background color which gave better contrast. Plaques so treated remained in excellent condition for several months. The increased intensity of the red color is probably due to precipitation of the dye *in situ,* since in the test tube solutions of the dye precipitate in the presence of mercuric chloride.

Results. The results in Table I show that the number of plaques formed with VEE and EEE virus was approximately inversely proportional to the dilution plated and that approximately the same satisfactory precision was indicated as in the results reported for the monolayer technic of Dulbecco and Vogt (7). In comparative studies of the suspended cell assay technic, and the monolayer assay procedure, 5 times more VEE virus was detected by the former method; the sensitivities of the 2 technics were equal for EEE virus.

Since virus was inoculated on the surface of the plates in this modification of the suspended cell plaque technic, it was reasoned that the resulting plaques should provide the proper conditions for quantitative replication. The results (Fig. 2) showed that except for plaques on the rim of the plate, which the velveteen did not touch, quantitative replication was achieved. The replicas of the already large VEE plaques were considerably larger than the original plaques (Fig. 2), partly because the plaques of this virus characteristically continue to enlarge on prolonged incubation. In contrast, the plaques of a mutant of VEE virus(8) are minute, and, like the wild type (r+)T-even coliphages, do not increase in size after 48 hours' incubation; they, therefore, replicated with minimum enlargement. After 3 replications, the plaques of the parent VEE virus became very large, probably because of spread of liquid and virus by the velveteen, which had become wet. If more than 3 replications are desired with satisfactory results (*e.g.,* for testing for host range mutants or recombinants), one can use as many as 3 velveteens on the same master plate with 3 replicas made from each.

Certain preliminary experiments showed that, as in mouse neutralization tests, EEE and VEE virus did not cross react in suspended cell plaque neutralization tests in which single virus particles failed to form plaques on antiserum-containing plates. These results permitted us to test whether replication of plaques was feasible for the screening of different antigenic types, such as might be used in recombination experiments.

Both VEE and EEE viruses were placed in discrete spots on the surface of agar plates containing the suspended chick fibroblasts. After 48 hours' incubation, the master plates containing plaques of both viruses were replicated in turn on separate plates containing approximately a 1/400 final dilution per plate of EEE or of VEE monkey antiserum, respectively. The plaque neutralization

TABLE I. Proportionality between Number of Plaques and Concentration of Virus.*

Virus strain	Dilution	No. of plaques on each plate	Avg No. plaques per plate	Titer per ml
VEE	4×10^{-7}	30; 35; 30; 21	29	5×10^{8}
	2×10^{-7}	14; 15; 15; 18	16	6×10^{8}
	1×10^{-7}	3; 6; 1; 5	4	4×10^{8}
EEE	4×10^{-7}	75; 38; 56; 60	57	9×10^{8}
	1×10^{-7}	15; 8; 12; 15	13	9×10^{8}

* 0.15 ml of appropriate virus dilution was inoculated on each 60-mm Petri plate.

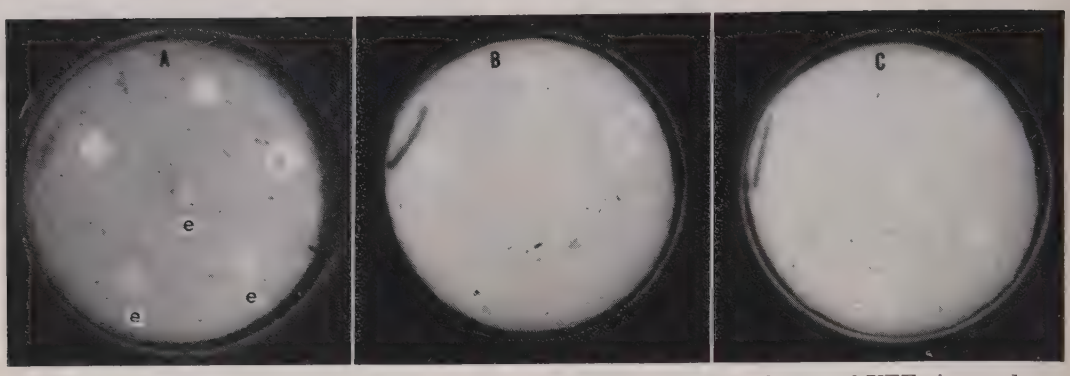

FIG. 2. Replication of virus plaques. A. Master plate showing clear plaques of VEE virus and smaller, turbid plaques of EEE virus. Latter are difficult to photograph and have been marked with an "e" beneath each plaque. B. Replica of A on plates containing EEE antiserum. C. Replica of A on plates containing VEE antiserum.

titers of the undiluted antisera were previously found to be over 1/1000 for each. The results of this experiment are shown in Fig. 2. The master plate shows the plaques of both viruses. EEE virus plaques are smaller and more turbid in appearance, and more difficult to see than are plaques of VEE virus by the suspended cell technic. By the monolayer technic, only size distinguishes the 2 virus strains. On plates containing VEE virus antiserum, the EEE plaques were quantitatively replicated in their expected geographical position, with the complete exclusion of the VEE virus plaques. Corresponding results were obtained for VEE plaques replicated on plates containing EEE virus antiserum.

The results of the above experiments suggest that replication of virus plaques for various purposes is feasible.

Discussion. The choice of the method finally selected for plaquing the viruses for replication was made after the standard monolayer method and Cooper's first suspended cell method(5,9) were found to be unsatisfactory. In the former no plaques could be replicated. The latter procedure involves mixing the virus and cells with agar before pouring it as an overlay. A poor efficiency of replication of viral plaques was obtained (20%-50%) using this assay method. Apparently, the viruses in the plaques beneath the surface either in the monolayer or in Cooper's first suspended cell procedure do not diffuse in sufficient amounts to the surface to permit quantitative replication. Cooper's second suspended cell method, which involves the infection of cells from the inoculum placed on the agar surface, was therefore modified to make it sensitive and precise for quantitative work and to make quantitative replication of plaques feasible. The present replication technic was developed with small Petri plates to illustrate the feasibility of the technic. Some of the disadvantages of the method stemming from formation of larger, overlapping plaques on the replicas could be minimized if larger plates were used. Similarly, means could be devised to place a removable artificial inside rim in the Petri dish so that plaques would not form on the true rim of the plate; the velveteen replication could then be made quantitative.

Summary. One of the 2 suspended cell plaque technics described by Cooper(5) has been modified so that its sensitivity and precision for plaque assay of VEE and EEE virus are satisfactory for general purposes. In addition, the modified suspended cell technic permitted nearly quantitative replication of virus plaques. Feasibility of the method in simulated systems for screening of host range or antigenic type recombinants (or mutants) was demonstrated.

The excellent technical assistance of Mr. Delbert Davisson in this work is gratefully acknowledged.

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Response of Growing Rats to Diets Varying in Magnesium, Potassium and Protein Content. (26757)

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Hypomagnesemia accompanied by convulsions is a disturbance which occurs sporadically in ruminants grazing on young, rapidly growing forage(1). A relationship has been observed in the field between nitrogen content of the grass on which cattle graze and incidence of tetany(2). High potassium diets(3) and diets high in protein and potassium(4) reduce serum magnesium and increase fecal excretion of magnesium in sheep. There is retention of calcium in the viscera and kidneys of rats during magnesium deficiency(5,6) and in the yellow elastic fiber of the endocardium, aorta and jugular vein in calves fed low-magnesium diets(7). Colby and Frye(8,9) reported an increase in severity of magnesium deficiency in rats fed high calcium, high potassium or high protein (50%) diets. It was desired to study the effects in rats of high potassium and/or high protein diets on growth, blood electrolytes and pathological anatomy of low or normal dietary magnesium.

Methods. Weanling albino rats of the Cornell strain were used in a factorial arrangement of treatments. Rats were kept in individual wire-mesh cages. Diets (Table I) were provided *ad libitum* and distilled water was available at all times. Feed consumption and weight gains were recorded weekly. One-half of the rats from each group were sacrificed after 28 days and the remainder after 56 days on test by ether anesthesia. Blood was collected by heart puncture into heparinized tubes for serum calcium and magnesium and whole blood potassium determination. Whole blood potassium was determined in duplicate after some modifications of the method of Mosher(10). Five tenths of one ml of whole blood were diluted 1:100 with distilled water for analysis. Serum calcium and magnesium were determined by using a modification of the method of Walser(11). The following organs and structures from each rat were prepared for histological examination: proximal epiphysis of tibia, parietal bone, brain (transverse section through thalamus and cerebellum), thyroid, parathyroid, adrenals, esophagus, trachea, stomach (esophageal and fundic sections), duodenum, jejunum, colon, liver, pancreas, spleen, bone marrow, lymph glands, kidneys, ureter, urinary bladder, skin, myocardium, skeletal muscle, lungs; cross section through hilus, including lungs, bronchi, aorta, mediastinum and thymus. Bones were fixed in 10% formaldehyde and soft tissues in Bouin's fluid. Decalcification was with Perenyi (5 parts 100% HNO_3 and 95 parts 80% ethyl alcohol). Samples were paraffin-embedded, sectioned at 6 micra and stained with H and E stain.

Results and discussion. The data for growth, feed consumption and efficiency of

* The senior author was an FAO Fellow during the period Sept. 1, 1959—March 1, 1961. Present address: Estacion Exp. Balcarce, Balcarce, Buenos Aires, Arg.

TABLE I. Composition of Control Diet (Diet 1).*

Component	%
Casein	12
Corn oil	5
Corn sugar (cerelose)	72
Cellulose (Solka-floc)	1.25
Vitamin premix†	5.00
Mineral premix‡	4.75

* Diets 1, 3, 5 and 7 contained 12% casein (10% protein); diets 2, 4, 6 and 8 contained 24% casein (20% protein). Diets 1, 2, 5 and 6 contained 240 mg magnesium/kg; diets 3, 4, 7 and 8 contained 24 mg/kg. Diets 1, 2, 3 and 4 contained 2033 mg potassium/kg; diets 5, 6, 7 and 8 contained 8132 mg/kg.

† Mixed with cerelose so that 50.0 g were required to supply the following vitamins for 1 kg of diet: vit. A, 4200 I.U.; vit. D, 135 I.U.; vit. E, 120 mg; vit. K, 0.2 mg; thiamine, 2.5 mg; riboflavin, 5.0 mg; pyridoxine, 2.4 mg; niacin, 30.0 mg; calcium pantothenate, 16.0 mg; choline chloride, 1500 mg; vit. B_{12}, 10 μg.

‡ Composition (units/kg of diet): Ca $(H_2PO_4)_2 \cdot H_2O$, 22 g; KH_2PO_4, 7 g; $CaCO_3$, 12 g; NaCl, 1.5 g; MgO, 0.4 g; $MnSO_4 \cdot H_2O$, 0.3 mg; $FeSO_4$, 0.25 g; $CuSO_4 \cdot 5H_2O$, 0.30 g; $ZnCO_3$, 0.045 mg; KI, 0.10 mg; Na selenite, 0.40 μg.

feed utilization are summarized in Table II. Low dietary magnesium (24 mg/kg) resulted in a highly significant ($P<0.01$) decrease in daily gain, feed consumption, efficiency of feed utilization. High dietary potassium (8132 mg/kg) had no effect on daily gain and feed consumption during the first 28 days but resulted in a highly significant reduction ($P<0.01$) in both after 56 days. There was no effect of level of dietary potassium on efficiency of feed utilization nor were there any potassium $\times$ protein interactions on any of these 3 criteria. High dietary protein (20%) resulted in a highly significant increase ($P<0.01$) in daily gain and efficiency of feed utilization after 28 days. This effect on daily gain but not on feed utilization was maintained after 56 days. There was a significant ($P<0.05$) magnesium $\times$ protein interaction in terms of daily gain. Mean daily gain of rats fed the low magnesium-low protein (10%) diets (3 and 7) was 0.98 g for the first 28 days and 0.76 g after 56 days. The corresponding values for those fed the low magnesium-high protein diets (4 and 8) were 1.15 and 0.65 g. However, a positive response to high protein occurred when dietary magnesium was adequate (2.30 *vs.* 1.62 g daily gain after 28 days and 1.97 *vs.* 1.52 g daily gain after 56 days) demonstrating that inadequate dietary magnesium prevents maximal utilization of protein for growth.

Blood values are shown in Table III. Low dietary magnesium decreased blood serum magnesium and calcium ($P<0.01$). Blood serum magnesium was affected by a highly significant ($P<0.01$) magnesium $\times$ protein interaction manifested in the rats sacrificed after 28 and 56 days on test. There was no

TABLE II. Effect of Dietary Magnesium, Protein and Potassium Level on Performance of Growing Rats.

Diet No.	Diet Designation*	Daily gain (g)§ 28†	Daily gain (g)§ 56‡	Daily feed (g)‖ 28†	Daily feed (g)‖ 56‡	Feed/g gain (g)¶ 28†	Feed/g gain (g)¶ 56‡
1	HMg-LP-LK	1.72	1.53	9.9	10.7	5.9	7.1
2	HMg-HP-LK	2.25	2.08	9.7	10.4	4.5	5.0
3	LMg-LP-LK	1.07	.90	8.3	9.1	8.0	10.7
4	LMg-HP-LK	1.23	1.09	7.8	8.6	7.5	8.4
5	HMg-LP-HK	1.52	1.50	9.0	9.6	6.2	6.5
6	HMg-HP-HK	2.35	1.85	9.8	10.2	4.3	5.6
7	LMg-LP-HK	.88	.61	8.0	7.8	9.8	14.2
8	LMg-HP-HK	1.06	.61	7.5	7.5	7.8	16.3

* HMg = 240 mg Mg/kg diet; LMg = 24 mg Mg/kg diet; LK = 2033 mg K/kg diet; HK = 8132 mg K/kg diet; LP = 10% protein; HP = 20% protein.

† Each value represents mean of 8 rats fed 28 days.

‡ Each value represents mean of 4 rats fed 56 days.

§ Significantly increased by high protein ($P<0.01$); significantly decreased by low magnesium ($P<0.01$) and significant magnesium $\times$ protein interaction ($P<0.05$) at 28 days. At 56 days same, plus significantly decreased by high potassium ($P<0.05$).

‖ Significantly decreased by low magnesium ($P<0.01$) at 28 days. At 56 days same, plus significantly decreased by high potassium ($P<0.05$).

¶ Significantly increased by low magnesium ($P<0.01$) at 28 days. At 56 days same, plus significantly decreased by high protein ($P<0.01$).

TABLE III. Effect of Dietary Magnesium, Protein and Potassium Level on Serum Calcium and Magnesium and Whole Blood Potassium of Growing Rats.

Diet		Serum Ca (mg %)§		Serum Mg (mg %)‖		Whole blood K (mg %)¶	
No.	Designation*	28†	56‡	28†	56‡	28†	56‡
1	HMg-LP-LK	8.2	9.7	3.2	2.6	206	211
2	HMg-HP-LK	8.4	9.5	2.8	2.5	197	219
3	LMg-LP-LK	6.7	6.9	1.0	.9	224	195
4	LMg-HP-LK	7.2	6.7	1.0	.9	221	220
5	HMg-LP-HK	8.2	9.9	2.5	2.6	220	217
6	HMg-HP-HK	8.2	9.3	2.6	2.6	224	222
7	LMg-LP-HK	7.0	7.4	1.6	1.8	211	190
8	LMg-HP-HK	7.1	7.2	1.6	1.6	217	195

* HMg = 240 mg Mg/kg diet; LMg = 24 mg Mg/kg diet; LK = 2033 mg K/kg diet; HK = 8132 mg K/kg diet; LP = 10% protein; HP = 20% protein.

† Each value represents mean of 4 rats fed 28 days.

‡ Each value represents mean of 4 rats fed 56 days.

§ Significantly reduced by low magnesium ($P < 0.01$) at 28 and 56 days.

‖ Significantly decreased by low magnesium ($P < 0.01$) and significant magnesium × potassium interaction ($P < 0.01$) at 28 days. Same plus significantly increased by high potassium at 56 days.

¶ Significant magnesium × potassium interaction ($P < 0.05$).

effect of high potassium diets on serum magnesium when dietary magnesium was adequate. However, mean serum magnesium of rats fed low magnesium-normal potassium diets (3 and 4) was 1.0 mg% after 28 days and 0.9 mg% after 56 days. The corresponding values for those fed low magnesium-high potassium diets (7 and 8) were 1.6 and 1.7 mg% indicating an inhibition by potassium of the reduction in serum magnesium associated with consumption of a magnesium-deficient diet. Typical magnesium deficiency symptoms were observed, however, in all 4 treatment groups (diets 3, 4, 7 and 8). Symptoms included reddening of the ears and paws and convulsions lasting 1-5 minutes culminating in death in 5 rats.

Histological examination revealed slight to severe nephrocalcinosis to be present in rats representing all groups as early as 28 days. This condition has been previously reported to occur in rats fed adequate diets. Otherwise no lesions were found. It is of interest that no tissue alterations associated with treatment appeared even after 56 days on test despite the fact that gross magnesium deficiency symptoms appeared as early as the first week.

Summary. Dietary magnesium, potassium and protein interactions were studied in weanling rats. Growth rate, efficiency of feed utilization, and blood serum magnesium and calcium levels were significantly lower in rats fed a magnesium-deficient diet than in those fed a similar magnesium-adequate diet. A diet containing 4 times as much potassium (8132 mg/kg) increased the serum magnesium level 60% after 28 days (1.0 *vs.* 1.6 mg%) and 90% after 56 days (0.9 *vs* 1.7 mg%) indicating an inhibition by potassium of the reduction in serum magnesium associated with consumption of magnesium-deficient diet. Typical gross magnesium deficiency symptoms, including reddened ears and paws and convulsions lasting 1-5 minutes were observed, however, in all groups fed low magnesium diets regardless of potassium level. Diets containing 20% protein produced significantly greater growth rate than those containing 10% in the presence of adequate magnesium but not during magnesium deficiency. No histological lesions associated with treatment occurred even after 56 days on test despite the fact that gross magnesium deficiency symptoms appeared as early as one week on test.

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Detection of Agents which Interfere with the Immune Response. (26758)

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Interference with antibody production by cytotoxic drugs, notably antagonists of nucleic acid constituents, has been reported by several investigators(1-8). Substances which exhibit this effect are of interest not only for the insight which they may provide into the immune process, but also for their possible applications to transplantation problems(9,10) and to autoimmune diseases(11).

Screening tests for such materials recently have been devised by Berenbaum(5) and by Sterzl(12) using antigens of bacterial origin. The method to be described herein is based on the agglutinin response in mice to administered sheep cells, and may therefore be regarded as a modification of the procedure used by Makinodan for investigation of radiation effects(13). As will be shown, the modifications which have been made appear to be essential for detection of the effects of drugs. The method as presently employed is simple, economical and capable of providing reproducible results.

Materials and methods. Mice. Specific-Pathogen Free (SPF) Swiss Bagg male mice* weighing 19-21 g were used.

Antigen. Sterile, washed sheep cells† were exposed for 10 minutes at 37°C to an equal volume of 1:10,000 tannic acid in buffered saline solution‡ collected by centrifugation, washed with buffered saline‡(7) and resuspended in the same medium.

Procedure. The initial inoculum of antigen consisted of 0.25 ml of a 30% suspension of cells per mouse, administered intravenously to groups of 10 mice. In the experiments reported herein a second dose of 1.0 ml of a 15% suspension of cells was administered intraperitoneally on the seventh day. The effects of this second dose have been found to be minimal, and in subsequent work it will be omitted. On day 13, the mice were decapitated and the pooled blood was allowed to clot. The serum was used for determination of hemagglutinins.

Hemagglutination. The hemagglutinin titer was determined by serial 2-fold dilution essentially by the method of Stavitsky(14) using 0.1 ml of a 1% suspension of tanned sheep cells per ml of saline-diluted serum. After preparation, the tubes were shaken and then allowed to stand for 18 hours at 5°. Agglutination was then scored 0, 1+ to 4+ described by Stavitsky(14).

Index. The score for each tube was multiplied by the appropriate exponent of the 2-fold dilution series, *i.e.*, a tube showing a 4+ agglutination at a dilution of 2^6 would have a value of 24. These values were then summed for each series. The index of drug effect was obtained as the ratio of these sums for the treated to the untreated control:

$$\text{Antibody Index (A.I.)} = \frac{\Sigma\,(S_1 + 2S_2 + 3S_3 + \ldots . nS_n)\,T}{\Sigma\,(S_1 + 2S_2 + 3S_3 + \ldots . nS_n)\,C}$$

where n refers to the exponent of the dilution

* Darrow Breeding Laboratories.

† Colorado Serum Co.

‡ Saline (0.85% NaCl) is mixed with an equal volume of buffer (29.9 ml M/15 KH_2PO_4 + 76.0 ml M/15 Na_2HPO_4).

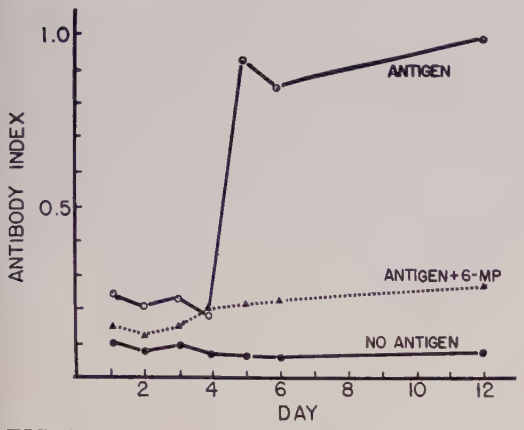

FIG. 1. Appearance of circulating antibody after administration of antigen. ●——● no antigen; ○——○ antigen on day 0; ▲----▲ antigen on day 0, mercaptopurine days 0, 1, 2, 3. Index as described in text.

(=tube number in the 2-fold series), S is the agglutination score for the tube and T and C refer to treated and control series, respectively.

The Σ_c for 28 trials = 175 ± 24.

A value of 0.5 or lower for the index was chosen arbitrarily as the limiting value for activity. However, it will be noted that this is removed from the control average by greater than 3 standard deviations.

Drug therapy. The drugs were given intraperitoneally at the estimated maximum tolerated doses (for initial screening) on 4 successive days, beginning at the time of the first antigenic stimulus. 6-Mercaptopurine (75 mg/kg) was included as a positive control in each experiment, as was a control without antigen or therapy.

Results. The results of hemagglutinin titers determined daily on groups of control mice and mice receiving therapy with 6-mercaptopurine (75 mg/kg/day) are presented in Fig. 1. For the first 4 days the titers were not significantly higher than those of the controls. This is in agreement with the findings of Makinodan(13). The rapid rise in titer on the fifth day is more abrupt than that reported by the previous author, but may be a consequence of the much greater antigenic stimulus provided in the present test. Antibody production is seen to be almost completely suppressed over the period studied when 6-mercaptopurine was administered during the first 4 days.

The relationship between antigenic stimulus, dose of drug and response was explored in a Latin square type of experiment, the results of which are presented in Table I. It will be seen by following each row that suppression of the response increases with increasing dosage of the drug. However, an increase in the antigenic stimulus resulted not in less, but, in fact, a considerably greater suppression. The rather high antigenic stimulus chosen for routine work appears well-adapted to the demonstration of the effects of drugs.

TABLE I. Relationship between Antigenic Stimulus, Dose of Mercaptopurine and Response.

		Dose of mercaptopurine, mg/kg/day × 4			
		0	8.33	25	75
		Index			
Sheep cell suspension*	.11	.78	.74	.50	.46
	.33	.78	.80	.60	.33
	1.0	1.00	.80	.61	.30
	3.0	1.06	—	.04	0

* In proportions of the standard inoculum (1.0) as given in the text.

Time of administration of the drugs in relation to suppression also was investigated. The pertinent data are shown in Table II. Suppression diminishes as treatment is delayed. Even a 24-hour delay appears to have some effect. The effect of single *vs* multiple doses of mercaptopurine also was investigated. In one experiment the usual regime of 4 daily doses at 75 mg/kg gave an index of 0.17, doses of 150 mg/kg on days 0 and 1

TABLE II. Effect of Time of Initiation of Therapy on Suppression of Immune Response.

Time of start	Index
Day 0	.34
1	.46
2	.77
3	1.04
4	.95
Control ẽ therapy	1.00

Each group received only the primary dose of antigen, as described in the text, on day zero. Each of the treated animals was given 4 daily injections of mercaptopurine, 75 mg/kg/day beginning on day indicated.

TABLE III. Effects of Various Agents on Hemagglutinin Response.

Compound	Dose/day, mg/kg	Index
Control without antigen	— (25)	.14 ± .06
Purine analogs		
6-Mercaptopurine	75 (16)	.32 ± .06
6-Thioguanine	2 (4)	.18
Benzimidazole	100	.85
B.W. 57-322*	12.5	.86
	25	.37
	50 (4)	.34
	100	.30
B.W. 57-323*	12.5	.30
6-Methylthiopurine	100 (4)	.42
6-Propylthiopurine	100	.67
8-Azaguanine	50 (2)	.58
Pyrimidine analogs		
5-Bromouracil	500	.69
5-Fluorouracil	25	.84
5-Hydroxyuracil	500	.99
5-Nitrouracil	500	.89
5-Aminouracil	200	.48
2-Thiouracil	50	.61
4-Thiouracil	200	.48
2-Thiothymine	250	.60
4-Thiothymine	200	.67
2-Thiocytosine	100	.63
6-Azauracil	500	.48
6-Azathymine	400	.68
Antifolic acids		
A-methopterin	2 (4)	.49
B.W. 50-276*	7.5 (2)	.51
Miscellaneous		
Nitrogen mustard	10†	.30
	5	.72
Hydrocortisone	37.5	.57
Chlorambucil	7.5 (5)	.47 ± .02
Chloramphenicol	250.0	1.18
Actinomycin C	0.2	.61
Actinomycin D	0.1	.45
Urethane	200	.83

Figures in parentheses refer to No. of trials, where averages of several are presented. Where a substantial No. of trials was involved, stand. dev. has been given.

* B.W. 57-322 6-(1-methyl-4-nitro-5-imidazolyl) thiopurine
B.W. 57-323 2-amino-6-(1-methyl-4-nitro-5-imidazolyl) thiopurine
B.W. 50-276 2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine

† Toxic dose, 5 of 10 mice died.

gave an index of 0.47, and a single dose on day 0 of 300 mg/kg gave an index of 0.51, with 2 of the 10 animals dying of drug toxicity. A number of experiments have indicated that there is no advantage, for the purposes of this test, in extending therapy beyond the 4-day induction period.

Representative screening test results are presented in Table III. It will be observed that 6-mercaptopurine consistently gives a strong repression of the immune response during the period of observation. 6-Thioguanine appears on the basis of a smaller number of trials to be significantly more active than 6-mercaptopurine. The imidazolyl derivatives (B.W. 57-322 and B.W. 57-323) have activities comparable to those of the parent thiopurines. However, B.W. 57-322 appears to be active over a wider range of dosage, and at lower fractions of the maximum tolerated dose which is approximately 150 mg/kg for this dosage schedule. The apparent superiority of this substance in comparison with mercaptopurine has been observed also in maintenance of renal homotransplants in dogs (10). 6-Methylthiopurine but not 6-propylthiopurine appears to have significant activity. 8-Azaguanine, on the basis of 2 trials, would be scored as inactive despite other evidence that in some circumstances it is capable of producing suppression(15). Among the pyrimidine analogs, the apparent inactivities of 5-fluorouracil, the various 2-thiopyrimidines and 6-azathymine are in agreement with the findings of Sterzl(12). On the other hand, 6-azauracil showed borderline activity as did 4-thiouracil, and 5-aminouracil. Two folic acid antagonists, A-methopterin and B.W. 50-276 gave borderline-active results. Nitrogen mustard (HN2) was active only at a toxic dose, but chlorambucil gave very consistent if not very powerful suppression. Actinomycin D was scored as active.

Discussion. The present screening test was designed primarily to find substances which interfere with the inductive phase of the antibody response. For this reason therapy has not been extended to the phase of antibody production. But experiments concerned with the optimal time of therapy show that 6-mercaptopurine, in agreement with the findings of Schwartz(2) has little or no activity once antibody production is established. The selection of the time of the first antigenic stimulus as the optimal time for initiation of therapy is not necessarily at variance with that of Berenbaum(5) who used a different antigen and a single maximal

dose of the test drug 48 hours after administration of antigen.

Where comparisons are possible, the results reported herein are in general agreement with those of Sterzl(12) although some minor differences with regard to the activity of specific compounds will be observed. In the present test to date, the activity with folic acid antagonists has been of moderate dimensions in contrast to the marked effects reported by others(4,12).

Perhaps the most important observation reported here is that the effects of the drug increase with increasing antigenic stimulus. The failure to employ an adequate antigenic stimulus may explain some of the failures to find suppression with 6-mercaptopurine. In the absence of a clear understanding of the processes involved in the inductive phase of antibody formation, the rôle of 6-mercaptopurine remains obscure. However, the present results suggest that maximal drug effects are obtainable only when these processes are stimulated maximally. It is noteworthy that all the substances found active to date also are active antitumor agents in tests with transplantable tumors in mice. The reverse, however, by no means follows.

Summary. A screening test is described for materials which suppress the immune response during its inductive phase. This test is based on the formation of hemagglutinins to sheep red blood cells in mice. 6-Mercaptopurine, 6-thioguanine and their S-imidazolyl derivatives, B.W. 57-322 and B.W. 57-323, show high activity.

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Effect of Stress on Response to Fludrocortisone Excess in the Rat.* (26759)

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We have reported that in rats treated with desoxycorticosterone acetate (DCA)(1,2) or STH(3), stress increases the hypertensive and/or nephrosclerotic action of the hormone, an effect not noted with cortisol(4). Selye(7) has reported that immature unilaterally nephrectomized rats given 200 μg/day of 9α fluoro-hydrocortisol (fludrocortisone) and 1% NaCl solution to drink, develop severe nephrosclerosis and ascites, but not cardiac lesions, encephalopathy or visceral periarteritis. Blood pressure showed an elevation to hypertensive levels in many animals, but this was not sustained, declining premortally.

It seemed of interest to determine the influence of simultaneous exposure to stress

* This study was supported by a grant from USPHS.

upon the patho-physiologic effects of fludrocortisone, since the steroid is potent both as a glucocorticoid and as a mineralocorticoid.

Materials and methods. Forty-two immature female rats of the Holtzman strain were used. Group 1 consisted of 10 animals exposed to stress for 12 consecutive hours daily. Group 2 consisted of 10 animals given 250 μg/day of an aqueous fludrocortisone acetate† suspension subcutaneously. Group 3 consisted of 12 animals given both of the preceding treatments and Group 4 consisted of 10 untreated controls. All animals were unilaterally nephrectomized on the day preceding inception of treatment and received 1% NaCl solution to drink throughout the experiment. Purina Laboratory Chow was fed.

Stress was imposed by the electrostressor (5) which delivered a brief electric shock to the animals at 2 minute intervals for 12 hours consecutively. Since food and water was unavailable during the imposition of stress, it was withheld from all animals during such periods.

Blood pressure was measured in unanesthetized animals by a tail plethysmograph, and pressures above 150 mm Hg were regarded as hypertensive. The animals were killed with ether on the 39th day and various tissues were excised for weight and/or histologic examination. Lesions in the heart and kidney, and in the mesenteric and pancreatic arteries, were evaluated microscopically.

Results. Stress alone did not cause hypertension. Fludrocortisone caused a definite increase in blood pressure to hypertensive levels in many of the animals. On the 33rd day of treatment hypertension was present in one animal under steroid treatment and by the 39th day 7 of the 9 survivors were hypertensive. In animals under dual treatment, 4 of the 10 survivors were hypertensive on the 33rd day, but by the 39th day 3 of these had died and only 2 of the group were hypertensive. The representative blood pressures are given in Table I. It was evident, particularly in the last 2 weeks of the experiment, that although stress alone caused but slight growth retardation it enhanced that caused by steroid. Mortality was also greater among those under combined treatment and half died before the experiment ended, although only one animal was lost from the group receiving only steroid. The 6 animals which succumbed to dual treatment had extensive gastro-intestinal ulcers and large, brown adrenals, indicating that stress had been much more severe in them.

Organ weights indicated that whereas fludrocortisone caused considerable adrenal atrophy in non-stressed animals ($P<.001$), it did not do so in stressed animals. Among the latter the adrenals were actually slightly larger proportional to body weight than in animals exposed only to stress, indicating that fludrocortisone-induced adrenal atrophy was blocked. Stress neither caused involution of the thymus and spleen, nor augmented that caused by steroid. Both renal and cardiac hypertrophy were evident in steroid-treated rats, and again this was not significantly increased by stress. The data are given in Table I.

Microscopic examination revealed no instance of visceral periarteritis in any of the animals and but a single example of focal myocarditis, the latter in an animal receiving only steroid. The kidneys of animals receiving hormone and which had developed elevated blood pressure, showed extensive change. Glomerular sclerosis or hyalinization of capillary loops was frequently noted. Occasionally fibrinoid necrosis of the wall of the afferent glomerular arteriole had taken place, although curiously intrarenal arteries and arterioles other than at this site were not involved. Hyaline casts occupied many of the tubular lumens. The incidence and severity of lesions seemed to be slightly greater in steroid-treated rats exposed also to stress, but the difference in severity was only slight and it seemed inadvisable to attempt quantitation of severity from examination of a single section from each kidney. Interstitial nephritis and/or fibrosis was noted in 3 animals exposed only to stress. Renal lesions were absent from controls.

† The steroid was generously supplied by E. R. Squibb and Sons.

TABLE I. Body and Organ Weight and Incidence of Lesions in Rats Exposed to Stress and Fludrocortisone Treatment Singly and Together.

		Stress	Fludrocortisone	Fludrocortisone + stress	Controls
Mortality, %		0	10	50	0
Body wt, g	Initial	59 ± 1*	58 ± 1	58 ± 1	57 ± 2
	Final	162 ± 2	109 ± 3	90 ± 5	184 ± 5
Blood pressure, mm Hg	Day 22	127 ± 4	129 ± 5	129 ± 1	120 ± 1
	" 33	127 ± 3	130 ± 5	148 ± 8	124 ± 2
	" 39	125 ± 4	166 ± 6	156 ± 9	122 ± 1
Organ wt, mg/100 g	Adrenals	39.8± 1.4	23.6± 1.2	41.4± 3.9	33.8± 0.7
	Thymus	212 ± 15	39 ± 5	35 ± 12	229 ± 9
	Heart	345 ± 9	431 ± 9	479 ± 22	318 ± 6
	Spleen	390 ± 28	235 ± 9	240 ± 12	328 ± 10
	Kidney	1059 ± 33	1600 ± 53	1511 ± 46	1019 ± 19
Lesions (% incidence)	Myocarditis	0	11	0	0
	Periarteritis	0	0	0	0
	Kidney	33.3†	66.6‡	100	0

* Mean ± S.E. of mean. † Interstitial nephritis. ‡ Glomerulosclerosis.

The kidney lesions in steroid-treated rats were similar to those resulting from desoxycorticosterone treatment, but differed in several details. Hyaline casts were not particularly abundant and there was little interstitial inflammation, perhaps reflecting the anti-inflammatory effect of fludrocortisone. The absence of involvement of renal arterioles, excepting those at the vascular pole of the glomerular tuft in the present circumstances, also contrasts with the necrosis, sclerosis and sub-intimal hyalinization so common in the vessels of DCA-treated hypertensive rats. It should be noted however that the degree of hypertension attained by animals in the present experiment was only moderate.

Discussion. The response of animals to fludrocortisone on the one hand, and the modification due to simultaneous exposure to stress were both distinctive. Steroid treatment caused certain changes that stress failed either to cause or modify; among these were enlargement of the heart and kidney and atrophy of the spleen and thymus. On the other hand, inhibition of body weight, presumably a reflection of the catabolic potency of the steroid, was greatest in animals under dual treatment, although stress alone showed some effect. Finally the steroid caused alterations opposite to those of stress, *e.g.*, effect upon adrenal size; and, under the circumstances of this experiment, stress predominated when both influences were imposed. The marked growth impairment, adrenal hypertrophy, prevalence of gastrointestinal ulcers and high mortality noted in the group under dual treatment left little doubt that the combination was much more severely injurious than either influence singly. The fact that the adrenals in these animals were, in proportion to body weight, if anything even larger than among rats exposed to stress alone, conformed with the high mortality observed. The amount of hormone, while sufficient to block resting ACTH secretion, as indicated by adrenal involution in the group given only steroid, clearly did not block the pituitary of animals under combined treatment. Insofar as hypertension and cardiovascular disease were concerned the results were less clear-cut. At the second blood pressure reading, on the 33rd day, the percentage of hypertensives among the group under dual treatment was much greater than in the group receiving steroid alone; hence the average pressure of the group as a whole was higher. Unfortunately the subsequent high mortality among hypertensives in the latter group reduced both average blood pressure and incidence of hypertension. Furthermore even the animals surviving dual treatment were emaciated and listless, and it is not surprising that the blood pressure proved to be somewhat unstable. It would thus appear that hypertensive disease was too great an additional burden for animals exposed to

both stress and the catabolic action of fludrocortisone to withstand.

Since cortisone enhances the renal damage caused by DCA(6), the great augmentation of renal enlargement and renal pathology by stress in rats receiving large doses of that steroid(2) could be construed as indicating that stress caused the adrenals to secrete a cortisone-like glucocorticoid. The present experiment seems to weaken the force of this hypothesis since stress did not as dramatically enhance the nephrotoxic action of fludrocortisone, which possesses both mineralocorticoid and glucocorticoid activity in high degree. It must be emphasized however, that stress enhances the pathologic effects of DCA on the vasculature much more markedly at high dosage of hormone than at low(2), and perhaps the results might have been more striking had a higher dosage of fludrocortisone been employed. The finding of either interstitial nephritis or fibrosis in 3 of the stressed animals is interesting. Such lesions were absent from controls, and animals receiving only steroid. Seemingly therefore the lesions were not of natural occurrence in the strain and may have been caused by stress, but suppressed by the anti-inflammatory effect of fludrocortisone in the steroid-treated stressed group. The slight incidence, however, precluded definite conclusions as to etiology and pathogenesis.

Summary. The influence of stress upon the response to fludrocortisone was assessed. The steroid caused atrophy of the spleen and thymus and hypertrophy of the heart and kidney, changes which were neither induced nor modified by stress. Body growth was slightly retarded by stress and markedly impaired by fludrocortisone, but the effect of combined treatment was greatest. Although the steroid alone caused adrenal involution, adrenal hypertrophy was found in animals subjected to stress or to stress and steroid treatment together. Mortality was insignificant from steroid alone, but great among animals subjected also to stress. There was some indication that stress enhanced the hypertensive and nephropathic response to steroid treatment, but precise assessment of this effect was rendered difficult by high mortality in the group exposed to dual treatment and the prevalence of emaciation and lowered vitality among survivors.

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Antithyroid Effect of Barbarin (Phenylthiooxazolidone), A Naturally-Occurring Compound from *Barbarea.** (26760)

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The capacity of certain crucifers to produce goiter has been known for some time (1). A potent antithyroid compound, goitrin (vinylthiooxazolidone), has been isolated from the edible parts of some members of this family(2,3,4,11) but seems to be contained in significant concentration only in turnip and rutabaga. It is present as an inactive thioglycoside, progoitrin(5), from which goitrin is released by enzymatic hydrolysis. Goitrin has an antithyroid potency 33% greater than propylthiouracil (PTU) in man and 2% that of PTU in the rat(2).

Recently a related compound, barbarin (phenylthiooxazolidone), has been isolated

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TABLE I. Antithyroid Activity of Barbarin in Rats.

Test material	Dose, mg	No. of animals	Avg % uptake
Barbarin	10	2	10.4
"	25	2	8.3
"	50	2	5.5
PTU	0.2	1	8.3
"	1.0	2	3.9
Control*	—	2	18.5

* Vehicle only.

from various species of *Barbarea* and from *Reseda luteola*(6,7). This material is contained in quite high concentration as its thioglycoside, glucobarbarin, in the edible green parts of the plants, which have heavy and widespread distribution through northern Europe. They are consumed by livestock and in some areas the young plants are eaten in salads or as vegetables. For this reason it was considered of interest to determine the antithyroid activity of barbarin.

Materials and methods. Because of the small amount of barbarin available, only limited studies could be made. Holtzman rats weighing 250 g were fed a low-iodine diet for 7 days. They were then injected subcutaneously with the test substance suspended in 10% gum acacia in saline. One hour later, 0.1 μC I^{131} was injected intraperitoneally. The thyroids were removed and counted in a scintillation well-counter 4 hours after radioiodine administration.

Assays in man were made by the technic of Stanley and Astwood(8). Epithyroidal counts were made at intervals of 20 minutes following administration of a tracer dose of 10-20 μC I^{131} and thyroidal radioactivity plotted as percent of the administered dose against the square root of the time in minutes. Once the slope of the "accumulation gradient" thus obtained had become clear (usually approximately 2 hours), the indicated dose of test substance was administered orally. Counting was continued at 20 to 60 minute intervals for the next 6 to 7 hours and repeated again 24 hours after the initial radioiodine administration. Changes produced in the accumulation gradient were graded from 0 (no effect) to 5 (maximal suppression) according to the classification of Stanley and Astwood. In addition, the 24-hour uptake calculated from the accumulation gradient was compared with the actual 24-hour count(9).

Results and discussion. The results are summarized in Tables I and II. Barbarin had an antithyroid activity roughly 1% that of PTU in the rat. The antithyroid effect of barbarin in man was about 75-100% that of PTU. Only limited examination was made of PTU since this had been extensively studied by Stanley and Astwood(8). Our previous assays of this drug as well as in the present investigation have confirmed their results.

This study indicates that barbarin has a significant antithyroid action, as would be expected from its structure. In both man and rat it is approximately 50% as potent as goitrin. The consumption of considerable quantities of plants containing this compound might therefore be a factor leading to development of goiter in both man and ani-

TABLE II. Antithyroid Activity of Barbarin in Man.

Subject	Drug	Dose (mg)	Effect	Accumulation gradient Initial	Accumulation gradient After drug	24-hr uptake (%) Predicted(9)	24-hr uptake (%) Actual
Mi	Barbarin	10	0	.69	.69	20.0 ± 3.2*	22.4
Mo	"	25	0	.84	.84	23.9 ± 3.7	26.1
Be	"	50	4	.97	.00	27.2 ± 2.7	18.9
Je	"	50	2	1.65	.40	42.6 ± 3.1	22.2
Wo	"	100	2	1.77	.18	45.1 ± 4.8	33.9
Lo	"	100	0	.93	.93	26.2 ± 3.5	24.7
Mi	"	200	4	.78	.06	22.4 ± 2.3	16.1
Na	"	283	4	.74	.00	21.3 ± 2.0	14.5
Je	PTU	50	2	2.34	.32	55.1 ± 5.4	38.5
Sa	"	100	3	.90	.00	25.4 ± 2.3	16.1

* Mean ± S.D.

mals. The activity of barbarin in animals which might feed vigorously on the wild plant is unknown. It is unlikely, however, that enough barbarin would be transmitted to cow's milk to be a potential hazard in the human consumption of this milk(10).

Summary. The antithyroid potency of barbarin, a phenylthiooxazolidone obtained from various species of *Barbarea* and *Reseda luteola,* was assayed by its inhibition of radioiodine uptake in rat and man. In both species it was found to be approximately 50% as active as goitrin (vinylthiooxazolidone).

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Cytopathogenicity of Mumps Virus in Cultures of Chick Embryo and Human Amnion Cells.* (26761)

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Primary monolayer cultures of chick embryo (CE) and human amnion (HA) cells have been utilized in assay of a number of animal viruses. To our knowledge, cytopathogenicity of mumps virus for these cells has not been previously reported. In the present paper cytopathic changes are described which were induced in these systems by mumps virus.

Methods and materials. Cell cultures. Methods of preparation and maintenance of primary cultures of HA and CE cells have been described(1,2). HA cells were maintained on Enders' medium(1) and CE cells on a medium consisting of 2.5% heat inactivated calf serum, 1.5% chick embryo extract, 48% bovine amniotic fluid (BAF), and 48% Hanks' Balanced Salt Solution (BSS). For plaque assay 3% Difco agar in BSS was added to an equal volume of a solution of 10% inactivated calf serum and 90% BAF. Neutral red dye (final concentration 1:20,000 or 1:40,000) was added 5-7 days after inoculation of virus. Dilutions of virus were incubated with cells for two hours at 37°C prior to overlay with agar. *Virus.* Two strains of mumps virus were employed: a) Enders strain, passaged 47 times in the amniotic cavity and 6 times in the allantoic cavity of the embryonated egg; b) a strain which caused CPE in HA cells on primary isolation,‡ and was subsequently passed 8 times in these cells. *Serological tests.* Complement fixation, neutralization and hemagglutination inhibition tests with anti-mumps guinea pig serum§ and hemadsorption of guinea pig erythrocytes were performed by

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† Postdoctoral Research Fellow of National Foundation.

‡ This was one of several strains of mumps virus isolated in HA cells by Dr. S. Kibrick in this laboratory.

§ Obtained from Microbiological Associates, Inc., Washington, D.C.

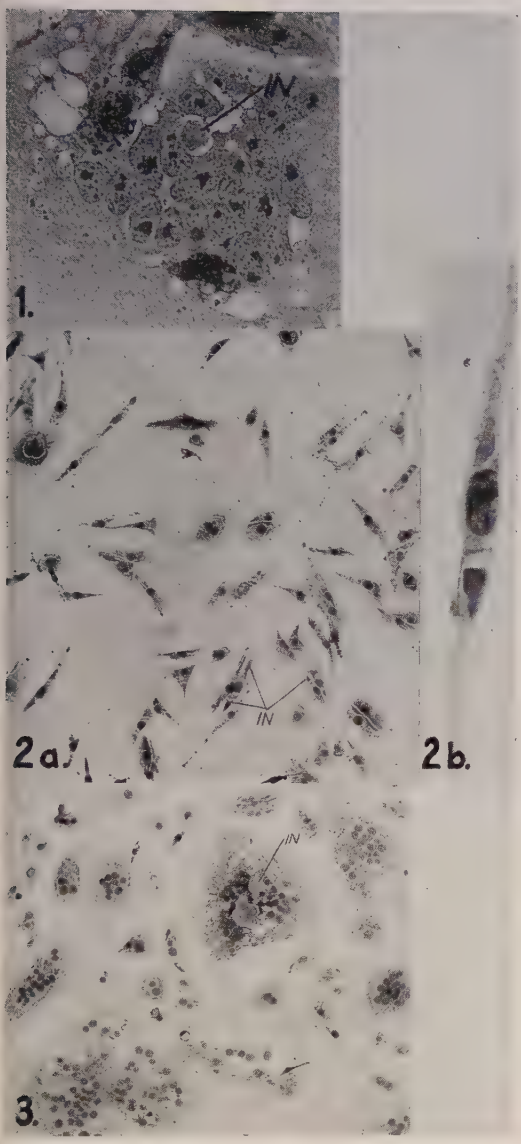

FIG. 1. Chick embryo cells 6 days after inoculation of mumps virus, demonstrating a giant cell with eosinophilic cytoplasmic viral inclusion body (IN). Mag. × 350. Hematoxylin and Eosin stain.

FIG. 2, a and b. Human amnion cells 8 days after inoculation of mumps virus demonstrating "spindle" cells with eosinophilic cytoplasmic inclusion bodies (IN). Mag. × 250 and 750. Hematoxylin and Eosin stain.

FIG. 3. Human amnion cells 6 days after inoculation of mumps virus; demonstrating giant cells with eosinophilic cytoplasmic inclusion bodies (IN). Mag. × 190. Hematoxylin and Eosin stain.

standard methods(3,4).

Experimental. Chick embryo cells. Cytopathogenic effect (CPE) induced by the Enders strain in CE cells was characterized by formation of multinucleated giant cells (Fig. 1), increased cellular refractility, and eventually almost total destruction of the cells. Cytopathic changes were observed as early as 4-5 days after inoculation of larger quantities of virus and by the 10th to 12th day with minimal reacting doses (10^{-6} TCD_{50}/ml). Eosinophilic cytoplasmic inclusion bodies surrounded by haloes were apparent after fixation and staining. Similar CPE was obtained upon serial passage of fluids from infected cultures to fresh preparations of chick cells. Cytopathic effects were inhibited by addition of mumps antiserum. Concomitantly with cell destruction, complement fixing antigen and hemagglutinin for guinea pig, chicken and frog erythrocytes in low concentrations (1:8-1:32 dilutions of culture fluid) were demonstrated.

With agar overlay, viral plaques (1-2 mm in diameter) were observed 6-8 days after inoculation of virus, but the toxicity of the neutral red dye for chick embryo cells made this technic less reliable than infectivity assay by tube titration.

In CE cultures infected with the Enders strain hemadsorption of guinea pig erythrocytes was observed 12-24 hours before cytopathic changes appeared.

In marked contrast to the behavior of the chick adapted strain in CE cells the human amnion cell strain failed to induce CPE or adsorption of erythrocytes.

Human amnion cells. Infectivity titers of the human amnion cell strain in HA cells ranged between $10^{-5.5}$-$10^{-6.3}TCID_{50}$/ml. CPE was noted as early as the third day after inoculation and the end point was attained after 10 days. Two types of CPE were observed. The first was characterized by formation of spindle-shaped cells (Fig. 2a,b) and the second type by appearance of multinucleated giant cells (Fig. 3). Both effects were observed in the same cultures, though often one or the other predominated. Attempts by terminal dilution technics to isolate a clone inducing only spindle or giant cell formation were unsuccessful.

Viral plaques (1-3 mm in diameter) were observed in HA cell cultures under agar 5

days after inoculation of virus. Plaque margins were not sharply defined and there was a tendency for plaques to enlarge eccentrically, creating a comet-like effect. A satisfactory correlation was noted in comparative titrations by the plaque and end point dilution technics.

In HA cells infected with the amnion cell adapted virus, hemadsorption of guinea pig erythrocytes preceded appearance of cytopathic changes by 12-24 hours. Neither of these manifestations of viral activity were observed following addition of the Enders strain to HA cell cultures.

Discussion. Multiplication of mumps virus in cultures of chick embryo tissues and membranes has been reported by several investigators(5,6,7,8,9,10). None, however, described cellular destruction associated with viral increase with the exception of Taylor (9), who observed inhibition of cell migration from fragments of infected chick amniotic membrane. Taylor interpreted his findings as evidence of viral cytopathogenicity—an interpretation that has been questioned by other workers(7,11). The present experiments show that in monolayer cultures of chick embryo cells chick adapted mumps virus consistently produces extensive cellular degeneration.

One can visualize several conditions that might account for the failure of previous investigators to observe CPE in fragments of infected chick embryonic membranes which were employed in all these earlier studies. For example, the insusceptibility of this tissue in contrast to that of the embryo itself may depend upon innate resistance of the individual cellular components. But it is also to be considered that resistance of the membranal cells is determined by preservation of their natural arrangement and inter-relationships under the conditions of cultivation employed. When cells are dispersed by trypsin and propagated in monolayers, as in the cultures we have studied, such "community" or histological resistance may be lost and the susceptibility of individual cells may then be readily demonstrable. Some support for this hypothesis is, perhaps, afforded by unpublished experiments of one of us (JFE), in which no evidence of viral multiplication was obtained in suspended fragment cultures of human amniotic membrane inoculated with poliovirus Type I. In human amnion cell monolayers this agent, of course, multiplies readily and causes complete destruction of the cells.

Although not directly relevant to the observed discrepancies with chick adapted virus in chick tissue cultures it is apparent from our results and those of others that cytopathogenicity for a given cell system may also be determined by the adaptive status of the virus. In our hands infection of HA cells with the human amnion adapted strain was followed by extensive cytopathic changes terminating in cell destruction whereas chick cells proved entirely refractory to this agent, as were HA cells to the chick adapted virus. Takemoto and Lerner(12) likewise failed to demonstrate CPE in cultures of HA cells after addition of a chick-adapted mumps virus. Henle and Deinhardt(13,14), demonstrated the capacity of freshly isolated strains to multiply and induce CPE in human cell lines and monkey kidney cells in contrast to chick adapted strains which could not be serially propagated in these cells. Recently Brandt(15) found that of 4 strains of mumps virus that had been cultivated in chick embryos only 2 that had received the least number of passages in this host were capable of continuous multiplication in mammalian cell cultures.

The viruses of mumps and measles are at present considered to be quite unrelated. It is of interest, therefore, that the cytopathic changes induced by mumps virus closely resembled those produced by measles(1). Thus both giant cell and spindle cell formation were regularly seen in mumps infected HA cells along with intracytoplasmic inclusion bodies. The latter were first described by Brandt(10). Intranuclear inclusions so characteristic of measles virus infection, were, however, not observed.

Summary. Cytopathogenicity of 2 strains of mumps virus for primary cultures of chick embryo (CE) and human amnion (HA) cells was studied. The chick adapted Enders strain induced formation of multinucleated

giant cells and extensive cellular destruction in CE cells but did not visibly affect HA cells. Multinucleated giant and spindle-like cells were observed in cultures of HA cells infected with a low passage human amnion adapted strain, which did not visibly affect CE cells. With agar overlay technic viral plaques were obtained with both strains in susceptible cell monolayers.

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Toxic Effects of Potato Sprouts and of Solanine Fed to Pregnant Rats. (26762)

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During one phase of a long-time feeding study involving reproduction in rats(1), some of the animals were fed a potato diet containing an estimated 7% of sprouts (wet wt.). On this diet, several of the rats delivered dead pups or pups that died within 2 or 3 days. The adults showed no toxic symptoms except, possibly, a lactation deficiency, and successfully raised subsequent litters after the sprouts had been eliminated from the diet.

The present report describes studies undertaken to confirm the toxic effects of sprouts and to isolate and identify the toxic factor involved. Exploratory tests confirmed the toxicity of the sprouts, both in a synthetic basal diet and in ground lab chow, when they were fed at a 10% level. When a sample of sprouts was dried at 50°, ground, and extracted successively with petroleum ether and methanol, the toxic factor was found to have been concentrated in the methanol extract. The methanol extract was found to be rich in alkaloids (Mayer's test). Furthermore during preliminary attempts at fractionation of the components of the methanol extract followed by biological tests, it was noted that those fractions which were exposed to mineral acid treatment lost an appreciable proportion of their toxicity. The latter observations led to the hypothesis that a glycosidic alkaloid such as solanine might be the toxic factor involved.

The general toxicity of sprouted potatoes and of solanine is well documented(2-5). However the literature appears to contain no studies of the effects of sprouted potatoes or of solanine on reproduction of animals. Consequently, potato sprouts in the frozen state were acquired for further sprout feeding tests and for isolation of solanine for inclusion in the biological tests. Preliminary tests indicated that the aglycone solanidine was inactive, and the latter alkaloid was therefore not included in the studies reported below.

Isolation of solanine. Frozen potato

sprouts (6.12 kg) were allowed to melt overnight at room temperature. The weight of the melt was 2.247 kg. The weight of damp sprouts was 3.930 kg. The sprouts were dried in a forced-air oven at 65° for 24 hours and ground; weight of ground sprouts, 802 g. Extraction of the dried sprouts was effected essentially according to the procedure described by Prelog and Jeger(6). The dried sprouts (400 g) were macerated with occasional stirring with 2% acetic acid (2 l) for 30 hours. The marc was separated by suction filtration and washed with 2% acetic acid (four 500 ml portions). The filtrate and washings were combined and alkalinized to pH 11 with concentrated ammonium hydroxide. The basic solution was allowed to stand for 12 hours at room temperature followed by 2 hours in the ice chest, and the crude alkaloid was separated by centrifugation. After washing twice with 2% ammonia solution (60 ml), the crude alkaloid was dried in a desiccator overnight (weight, 6.3 g). Exhaustive extraction with ether in a Soxhlet extractor dissolved 1.08 g. The ether-insoluble solid was crystallized from 80% ethanol, yielding 1.18 g (0.038%) of crystalline solanine, m.p. 278° after sintering from 269°. Recrystallization from 80% ethanol yielded 738 mg (0.024%) of solanine m.p. 279° after sintering from 270°: $[\alpha]_D^{30}$ −58° (c 4.00 pyr.). Reported m.p. 285°, $[\alpha]_D$ −56.5, −60° (pyr.)(6). Extraction of the aqueous melt afforded only a trace of additional alkaloid.

Methods. Holtzman rats approximately 4 months of age were mated, one male to 4 females. As soon as pregnancy was indicated by increase in weight, the females were placed in individual cages and fed one of the following diets.

I	Basal	(ground commercial lab chow)
II	"	+ 10% ground frozen sprouts (wet wt.)
III	"	+ 30 mg/kg solanine*
IV	"	+ 40 mg/kg solanine*
V	"	+ 30 mg/kg solanine†

* Solanine obtained from Mann Research Laboratories, Inc., N. Y., and from L. Light and Co., Ltd., Colnbrook, Bucks, England.

† Solanine isolated from the frozen sprouts.

TABLE I. Effect of Potato Sprouts and Solanine on Survival of Rat Pups.

Group	No. litters	Pups Born	Pups Weaned	% pups weaned	No. zero litters*
I	11	115	95	82.6	1
II	9	83	42	50.6	5
III	10	100	31	31.0	6
IV	10	106	33	31.1	5
V	4	41	8	19.5	2

* Litters in which all pups died.

Some of the rats were on the test diets for only a few days before dropping their first litter. They were then kept on the test diet until they had a second litter. The rats ate the diet readily; no food consumption records were kept.

Results. The toxic effect of the test diets is indicated in the Table which gives, for each diet, number of litters, total number of pups born and weaned, percent of total pups weaned, and number of litters in which all pups died. Most of the deaths occurred within 3 days of birth and were evidently caused by starvation.

Group II contained 2 rats which raised one litter, and then a second litter while eating the sprout diet. None of the pups in the other 5 litters of this group survived to weaning age.

Group III contained one rat which successfully raised 2 litters on the test diet.

Discussion. The results of this feeding test indicate that the toxicity of potato sprouts to pregnant rats is due to the alkaloid solanine. The exact nature of the toxicity has not been determined. However, it was observed that the pups that died did not have milk in their stomachs. One attempt was made to foster nurse control pups on a solanine-fed mother. This was unsuccessful and we did not have another opportunity with these animals. There was no noticeable effect on the adult males, either on body weight or breeding activity.

The evidence suggests that the alkaloid may be toxic for certain functions such as lactation, possibly by virtue of an anti-hormone effect. It also suggests that there is individual variation in susceptibility, since 3 of the rats were able to raise, to weaning, 2 litters while consuming a test diet. This is

in contrast to the 18 litters in which all pups died.

Summary. Potato sprouts are shown to be toxic to pregnant rats when fed at a level of 10% of the diet. When the potato alkaloid solanine was incorporated into the diet at a level approximating the concentration in the sprout diet, similar effects were observed. All of the pups in 18 of 33 litters born of rats eating the test diet died before reaching weaning age. Only one of 11 control litters was lost.

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Effect of Nephrectomy and Splanchnicectomy on Plasma Disappearance of Labeled Insulin in the Rabbit.* (26763)

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Studies have revealed that the disappearance rate of insulin-I^{131} from the plasma is influenced by a number of factors: plasma binding(1), state of hepatic and renal function(2,3), route and rate of administration (4), size of the endogenous insulin pool(5), and previous administration of heterologous insulin(6). Determinations based on accumulation of isotope in the liver and kidney after injection of insulin-I^{131} have implicated these organs in the process of insulin degradation(2). Such evidence is open to question since the concentration of radioactivity in these organs does not necessarily have any direct bearing on concentration of insulin. Berson *et al.*(1) and Scott *et al.*(7) have noted that the labeled material disappears from the plasma according to a curve with at least 2 different time components. On the basis of electrophoretic studies these authors have reasoned that the initial fast component of the disappearance is most representative of true insulin while the second slower component may represent the disappearance of some labeled product other than insulin. Bolinger and Slinker(3) studied the parameters of the slower component of the disappearance curve in rabbits and showed that the status of hepatic and renal function affected this component. The following study is designed to study the characteristics of the rapid component of the disappearance curve of labeled insulin in the rabbit and if possible arrive at a quantitative measure of the importance of the liver and kidney in this process. An attempt is made to define the system more specifically by including values obtained for biologic assay of insulin activity in the plasma, in order to arrive at a better estimate of the endogenous insulin pool.

Methods. Adult white rabbits were fasted for 48 hours. After light anesthetization with pentabarbital blood samples were drawn and I^{131} labeled insulin (.06 Unit) was injected intravenously. Blood samples were drawn by cardiac puncture at 10, 20, 30, 45, and 60 minutes. Plasma glucose was determined by the glucose oxidase method(8). Radioactivity was determined on both trichloracetic acid precipitates and supernatants of the plasmas, using a well counter and expressed as fraction of dose injected. Electrophoresis

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was carried out on paper strips using the Spinco apparatus. Insulin-like activity of the plasma was assayed using the rat diaphragm according to the method of Willebrands and Groen(9). Since the method does not discriminate between the small changes in plasma insulin activity occurring after the injected test dose and at the different sampling times, activity was determined on a pool of the timed plasma samples taken from each animal and the level of insulin activity assumed to be a constant during the test procedure. This assumption appeared valid in view of the insignificant changes in blood glucose after injection of 0.06 Unit of labeled insulin.

The animals were divided into 3 groups: Group I were normal animals; Group II, animals in which the kidneys were removed acutely just before the test procedure; Group III, animals in which splanchnic circulation was completely occluded acutely just before the test procedure. A group of sham operated controls showed no striking deviation from the normal.

Results. Assuming the steady state, rate of consumption of insulin can be theoretically defined:

$$R = k_1 S C$$

where R is consumption of insulin (milli-units/min/kg)

k_1 is a first order rate constant for disappearance of labeled insulin from the plasma (min^{-1})

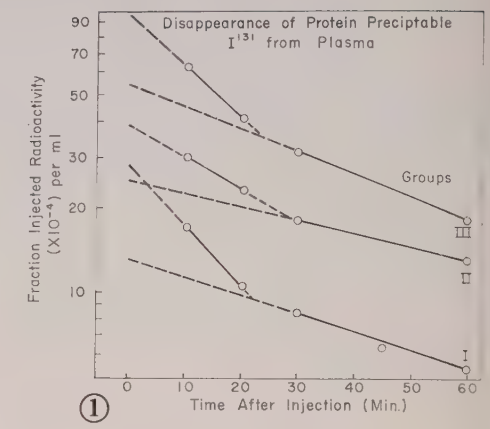
S is the space of distribution of the labeled insulin (ml/kg)

C is concentration of insulin in the plasma as determined by biological assay (milli-units/ml). This is assumed to be constant for each animal.

The product k S expresses the volume of body fluid (per kg) which is cleared of labeled material per minute.

The curve of disappearance of the labeled material from the plasma can be resolved into at least 2 first order components (Fig. 1). The following determinations are made on the rapid component of the curve during the first 20 minutes.

Space of distribution. In the normal rabbits the labeled material distributed itself over a volume of about 18% of total body volume, approximately that of extracellular fluid (Table I). Nephrectomy produced an insignificant decrease in the space while splanchnicectomy produced a marked decrease to 4.7%.



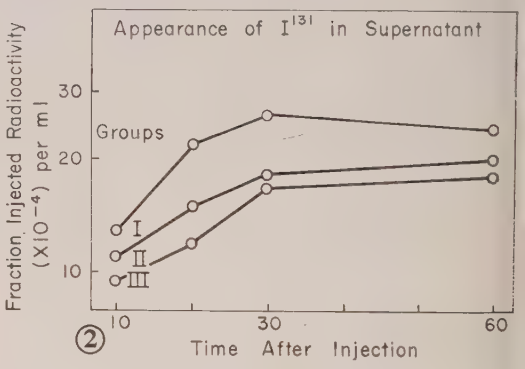


FIG. 1. Mean curves for disappearance of TCA precipitable radioactivity for each group. Actual curves are shown with solid lines and extrapolated curves with dotted lines.

FIG. 2. Mean curves for disappearance of TCA non-precipitable radioactivity for each group.

Disappearance constant in the normal animals was 0.0471 min^{-1} corresponding to a half time of 15 minutes. Nephrectomy produced a significant decrease in the disappearance constant while the rate in the splanchnicectomized animals was not different from normals.

The disappearance constant can not, however, be considered as a true measure of rate of disappearance of insulin since the space of distribution in each case is different. Thus a better measure of the true rate of disap-

TABLE I. Parameters of Plasma Disappearance of Labeled Insulin.

Group	No.	Insulin space, ml/kg	Rate constant, k_1 (min.$^{-1}$)	Half life, min.	Insulin conc., 10^{-3} U/ml	Insulin* consumption, 10^{-3} U/min./kg	Clearance* k S, ml/min.
I	9	186 ± 55	.0471 ± .0139	15 ± 5.1	.78 ± .32	7.04 ± 4.22	8.5 ± 3.7
II	4	145 ± 19	.0289 ± .0045	25 ± 6.2	.82 ± .13	3.64 ± 2.06	4.2 ± .7
III	7	47 ± 16	.0470 ± .0163	17 ± 5.4	5.61 ± 2.55	10.57 ± 2.14	2.1 ± .7

$$\text{S.D.} = \sqrt{\frac{\Sigma x^2}{N}}.$$

* Means of groups are significantly different from each other by analysis of variance.

pearance of insulin is the clearance factor (kS). In normals this was 8.5 ml/min/kg and nephrectomy was followed by a decrease. Splanchnicectomy produced an even greater decrease.

Consumption of insulin (R) in the normal animals is 7.04 milli-units/min/kg. Consumption of insulin is decreased after nephrectomy but not after splanchnicectomy. Actual increased insulin consumption after splanchnicectomy may be only apparent since it is based on the striking increase in assayable insulin activity from the plasma. The increased values for biological activity may be a result of exclusion of the liver from the circulation which could alter the assay by affecting the plasma binding of insulin(10) or by permitting accumulation of amino acids giving a false high value for insulin like activity(11).

Rate of appearance of radioactivity in the supernatant is a reflection of rate of breakdown of labeled insulin. This is decreased in both Groups II and III (Fig. 2).

The serum from each blood sample was electrophoresed and radioactivity from each protein fraction assayed. During the first 20 minutes, used for determination of the disappearance curves, 95% of radioactivity was in the beta globulin fraction.

Discussion. Evidence for the assumption that serum precipitable radioactivity is representative of true insulin after administration of labeled insulin, is largely indirect, since no direct experiment has yet been designed to demonstrate this. It is based, first, on the finding that after addition of labeled insulin to the serum, both biological activity and radioactivity are recovered primarily from the beta globulin fraction(12). Of the protein bound radioactivity in this experiment, 95% is in the beta globulin fraction. Furthermore Scott *et al.*(7) showed that the disappearance of biological activity from the plasma paralleled the same rapid component curve as that of the radioactivity from the globulin fraction, having the same mobility as that of insulin. That there are 2 different disappearance curves for the radioactivity from the serum does not exclude the possibility that both are representative of true insulin, since insulin may exist in the plasma in various states of binding with the plasma protein fractions.

Splanchnicectomy causes the greatest decrease in quantity of plasma cleared of insulin per unit of time. The capacity of the organism to consume insulin, however, is not compromised because of the ability of the remaining renal circulation to remove insulin at a higher plasma concentration of insulin. Conversely the greatest impairment in insulin consumption is produced by nephrectomy. These data are consistent with an hypothesis that both renal and splanchnic circulations can function in the degradation of insulin, but that at a higher level of plasma insulin concentration, the renal circulation can assume all of this function, in spite of a decreased insulin clearance. A true evaluation of insulin degradation must take into account not only the disappearance rate of the labeled material but also insulin concentration and space of distribution.

Summary. The plasma clearance and consumption of I^{131} labeled insulin was determined in normal, nephrectomized and splanchnicectomized rabbits. The rate constant for plasma disappearance of labeled material was decreased by nephrectomy but

not by splanchnicectomy. The plasma clearance of insulin was decreased by splanchnicectomy but the remaining renal circulation was able to maintain the consumption rate of insulin at a normal level.

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Electron Microscope Studies of Intact Epithelial and Fibroblast Cell Surfaces.* (26764)

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The earliest electron microscopic studies of tissue cell morphology utilized whole cells grown in tissue culture on formvar-coated copper wire grids. This technic, as described by Porter *et al.*(1), allowed visualization of some cell structures not seen by light microscopy, but did not permit the extensive studies of cellular morphology afforded by ultrathin sectioning. Consequently, the above method has had limited use and only a few additional studies(2,3,4) have been reported. Almost all of these appeared prior to the present widespread use of tissue culture as an experimental approach to various problems.

Although whole cell preparations are too thick to allow resolution of most structures in the nucleus and cytoplasm, the cell surface is clearly seen. Microvilli and other structures which project from the cell surface at various angles usually appear in thin sections as disconnected parts while in the whole cell mounts their structural integrity is retained. For certain studies in progress in this laboratory it was desirable to determine: (a) Whether the surface features of different tissue culture cell types are similar or vary significantly from one cell line to another, and (b) whether the surface morphology of one cell line studied over a prolonged period of time remains constant or varies. The present paper compares the surface morphology of 5 cell types commonly used in tissue culture and describes certain modifications of technic affording reproducible whole cell preparations in which microvilli and other surface structures are well preserved and remain free of most extracellular debris.

Materials and methods. Tissue culture. Cell types used included chicken fibroblasts, mouse fibroblast ("L"), HeLa, Chang's liver and chicken monocytes. These cells were grown and maintained in stock flasks of various sizes and after suitable sheets had been obtained were trypsinized into suspension. The cell suspensions were then adjusted to contain the following cell concentrations: 1-2 million chicken fibroblasts or 600,000 "L",

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150,000 HeLa cells or 125,000 Chang's liver cells. HeLa and Chang's liver strains were obtained initially from Microbiological Associates. Chicken fibroblast cultures were prepared in this laboratory from embryonic tissue. Chicken monocytes were obtained as follows. The buffy coat was separated from adult chicken whole blood, washed and the total cell suspension of 4-5 million cells/ml inoculated directly into the Leighton tubes, because monocyte sheets are not readily removed and resuspended from flask cultures. All cultures contained penicillin 100 units/ml, streptomycin 100 μg/ml and mycostatin 50 units/ml.

Electron microscope grid mounts. Stainless steel 200 mesh grids were glued (scotch tape glue in xylene) to a 10 $\times$ 50 mm #1 glass cover slip. This surface was then used to pick up a 0.4% collodion membrane which was then air dried. Thus, a collodion film coated both the grid surface and the surrounding glass area overlapping to cover part of the opposite side of the cover slip. After drying, the entire grid-cover slip preparation was placed in the vacuum evaporator and coated with a thin carbon film.

Tissue culture cells growing on the coated grid were observed by light microscopy during the culture period and removed when cells were well spaced over the membrane but, of course, before continuous sheets were formed. At this point the glass cover slip was removed from the tube, washed in buffered salt (Hanks') solution and fixed for 90 seconds in 1% veronal-buffered osmium, pH 7.4(5). After fixation the cover slip grid preparation was washed in distilled water, air dried and metal shadowed with chromium. Micrographs were made with an RCA-EML electron microscope.

Results. Comparison of the microvilli and other surface features of the cells studied revealed striking morphologic differences and the specific structural features associated with a particular cell line remained constant unless variations were induced deliberately. Variations in age of culture, time-length of fixation or per cent of osmium used induced no significant morphologic changes. This does not mean that each cell was precisely like all others of its type but the differences observed were quantitative rather than qualitative.

TABLE I. Growth of Tissue Culture Cell Lines on Grids Coated with Formvar, Collodion and Carbon-Collodion Membranes.

Cell line	Type of membrane		
	Formvar	Collodion	Carbon-collodion
Chicken fibroblast	3+	3+	4+
Mouse fibroblast (L)	0	2+	4+
HeLa	2+	2+	4+
Liver (Chang)	0	2+	4+
Chicken monocyte	2+	2+	4+

4+ = normal growth, no rounded or degenerating cells.
0 = no normal cells, all cells rounded and/or degenerating.

The importance of present modification of methods for this type of preparation must be emphasized since there was marked variation in the ability of cells of different cell lines to grow on surfaces suitable for electron microscopy. Thus, formvar membranes allowed reasonably good growth of chicken fibroblasts as reported by Porter(1) but were completely unsuitable for the "L" cells which did not put out processes on this surface and remained in a rounded form. Under these latter conditions few microvilli were seen projecting from the cell surface, and degeneration of most cells occurred within 24 hours. The growth of the various cell lines with various membranes is presented in Table I.

The following paragraphs summarize the morphologic observations of several hundred cells of each type. These specimens were prepared in many separate experiments over a period of several months. In no instance did the prototype cell morphology vary significantly. For example, "L" cell preparations studied months ago and new preparations studied recently were entirely comparable as was true with all other cell types.

Strain "L", mouse fibroblast (Earle). This cell was characterized by its elongated appearance and a profusion of microvilli projecting from all surfaces (Fig. 1 and 2). No nuclear or cytoplasmic structure was ever visible. The microvilli were 1-10 μ long and 0.1-0.3 μ wide without a terminal bulb structure. Microvilli were more profuse in the

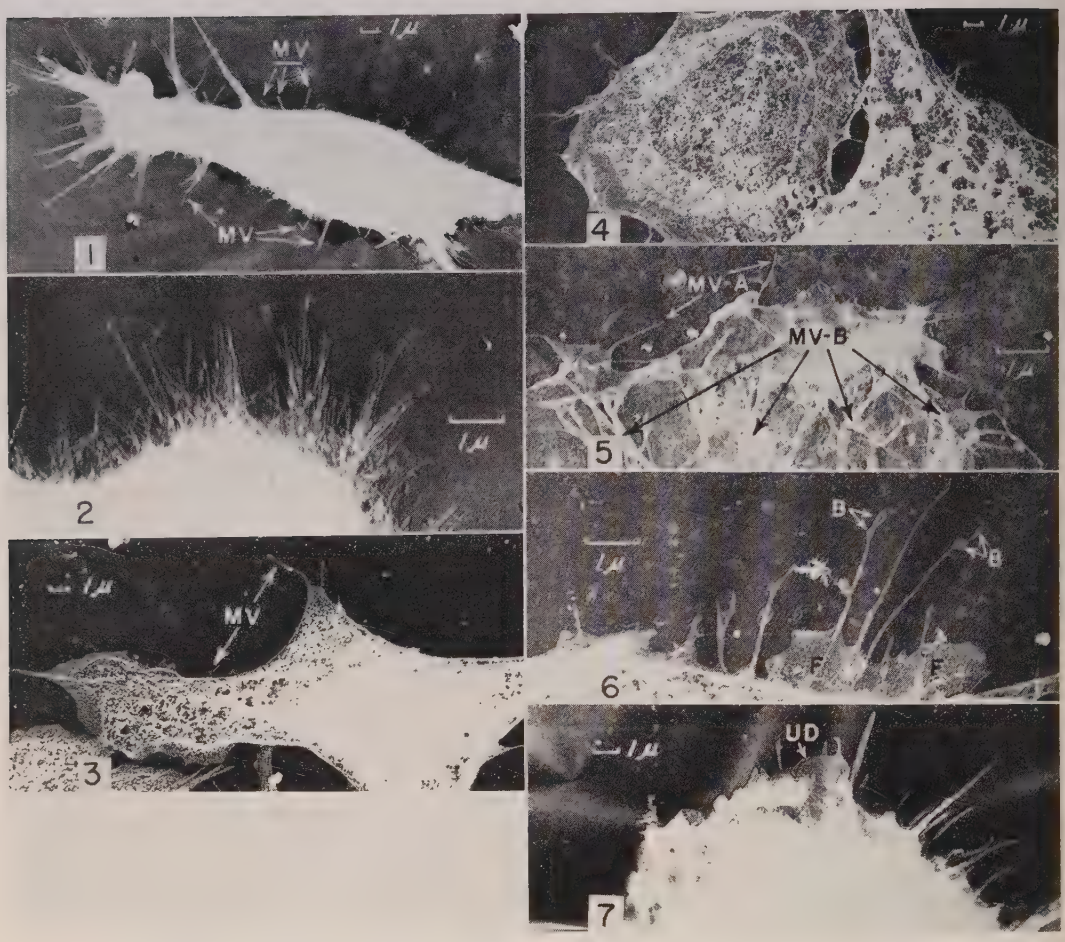

FIG. 1. Mouse fibroblast strain "L" (Earle). Only a few of the larger microvilli (MV) are specifically indicated. The cell body is always thick and opaque to the electron beam. Chromium shadowed. × 1600.

FIG. 2. A higher magnification of the surface of another "L" cell. The profusion of villi projecting from the surface is characteristic. Chromium shadowed. × 6400.

FIG. 3. Chicken fibroblast which has flattened so that some cytoplasmic structures are visible. The cell shape resembles the "L" cell but microvilli are numerically few. Chromium shadowed. × 1600.

FIG. 4. Two HeLa cells showing a few intracellular bridges. At this magnification only a few microvilli appear to be present. Chromium shadowed. × 1600.

FIG. 5. Higher magnification of a HeLa cell surface. MV-A indicates typical bulbless microvilli extending out from cell surface; MV-B locates masses of villi folded back onto cell body. Chromium shadowed. × 6400.

FIG. 6. Typical Chang's liver epithelial cell surface showing bulbous (B) microvilli which extend through or arise from a flap (F) of cell cytoplasm. Chromium shadowed. × 6400.

FIG. 7. Chicken monocyte. In this cell type villi appeared in patches and most of the visible surface was occupied by a membranous structure, seen here in part, probably corresponding to the undulating membrane (UD). Chromium shadowed. × 1667.

regions of cell processes but were present to some extent on all surfaces.

Fig. 1 shows the typical spindle shape which is similar to that of the chicken fibroblast in Fig. 3. The number of microvilli (MV) in Fig. 1 represent the minimum found with the "L" cell. Most cells of this type showed the profusion of microvilli seen in Fig. 2.

Chicken fibroblast. As previously observed by Porter *et al.*(1), this cell tended to flatten considerably so that internal cell

structure could be seen (Fig. 3). Microvilli were absent in most areas and only a few per cell were seen, usually concentrated in the region of the cell processes.

HeLa epithelial cell (Gey). This cell (Fig. 4 and 5) had an irregular shape exhibiting more variation in this regard than any of the cells studied. Internal structures of the cell, the nucleus and cytoplasm, were visible in about half of the cells. The surface was characterized by large numbers of microvilli which varied greatly in length but had a fairly uniform width of 0.05 to 0.2 μ. Occasionally HeLa microvilli terminated in a bulb but enlarged areas of the body of the villus have not been found similar to those noted for Chang's liver cells. The distinguishing feature of this cell was the folding back of the cell edge onto the cell body proper as seen in Fig. 5. This configuration was present in all the cells examined and conforms in general to the "ruffle edge" described by Lewis(6). Superficial examination of HeLa surfaces at low magnification (Fig. 4) would suggest that very few microvilli are present. A higher magnification micrograph (Fig. 5) shows that a few villi, MV-A, project out from the surface but many more, MV-B, are seen folded back onto the cell body. Although this surface configuration was found occasionally in the Chang's liver cells (described below) it was far less prominent than in the HeLa cells. The folding back of the microvilli was not found with the chicken fibroblast, the mouse fibroblast or the chicken monocyte.

Liver epithelial cell (Chang). In shape and amount of internal structures visible this cell resembled HeLa but examination of the surface at high magnification revealed two distinct differences. As indicated above, the microvilli did not tend to fold back onto the cell body although this was occasionally seen. More characteristic was the appearance of bulbous enlargements of the villi (Fig. 6B). Usually a single terminal bulb was present but in some instances 2 such enlargements were found. In addition, villi appeared to project through thin flaps or extrusions of the cytoplasm (Fig. 6,F).

Chicken monocyte. This cell generally assumed an oval or round shape and the cell body was opaque to the electron beam so that no internal detail was seen. The cell surface was characterized by microvilli appearing in patches and varying greatly in number, length and width. Fig. 7 illustrates the typical appearance of this cell type. Microvilli project out from one segment of the surface and the remaining surface area consisted of a smooth, scalloped membranous structure corresponding to the undulating membrane (UD) observed by light microscopy. It is of interest that micrographs of this cell invariably showed folding of the underlying carbon-collodion membrane to an extent not found with other cell studies. The monocyte is well known for its strong adhesion to surfaces on which it grows, which may account for the folding of the grid membrane.

Discussion. The present study demonstrates that the surface morphology of the 5 cell lines examined varies considerably from line to line particularly in regard to number and configuration of the microvilli. On the other hand, the surface structure of any given one of these 5 cell lines remains quite constant and reproducible when the tissue culture preparations are made in the manner described. The original method for whole cell electron microscopy(1) utilized chicken fibroblasts grown on formvar-coated copper mesh grids. This technic provides reasonably good results with chicken fibroblasts but is of little or no value for some of the other cell types. The mouse fibroblast strain "L", for example, will not grow normally on formvar and the other cell lines used have shown varying degrees of toxic or abnormal changes apparently due to the formvar, the copper or both. Carbon-collodion films on stainless steel grids allow cell growth comparable to that observed on glass.

The reproducibility of the cell surface structures of any of the 5 lines examined was very good in contrast to the marked variations found when the same cells were grown on either formvar or plain collodion. Blind tests to determine cell type on the basis of the surface morphology have demonstrated that the distinction between the mouse fibroblast, chicken fibroblast and chicken mono-

cyte is extremely easy and accurate. The ability to distinguish between the liver epithelial cell (Chang's) and HeLa is more difficult but can be accomplished if one scans a number of cells. It is not proposed that all tissue culture cell lines can be distinguished in this manner but knowledge of their surface features may be of some help.

Some of the features of cell surfaces described here can be seen and have been photographed in the light microscope. The undulating membrane and the patchy distribution of the microvilli in the chicken monocyte and the folding back of the HeLa cell edge correspond to similar structures seen in light micrographs and reported by various investigators(7,8). It would appear, then, that the present technics have not introduced an inordinate amount of cell distortion.

Summary. An improved method for the electron microscopic study of intact tissue culture cells is based on the use of carbon-collodion coated stainless steel grids upon which the cells are grown. Cell preparations are fixed with buffered osmium and metal shadowed. This technic has been applied to the study of 5 cell lines including mouse fibroblasts strain "L" (Earle), HeLa (Gey), liver epithelial (Chang), chicken fibroblasts and chicken monocytes. Distinctive surface features of these cell lines are described.

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The Tumor Necrotizing Effect of Lipoid A Component of *Escherichia coli* Endotoxin.* (26765)

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The endotoxins of gram-negative bacteria cause a large variety of primary and secondary reactions in susceptible hosts, including fever, the local and generalized Shwartzman reactions, hemorrhagic necrosis in experimental tumors, as well as alterations in non-specific resistance, immunological response, properdin levels, and dermal reactivity to epinephrine(1-5). Chemically, the endotoxins are complexes made up of polysaccharide, protein or peptide, and lipoid. It is the polysaccharide moiety that is responsible for serologic specificity. Recent studies suggest that the lipoid A component of this complex is pyrogenic, alters nonspecific resistance to experimental infection, and is responsible for enhanced reactivity of the rabbit to epinephrine(6,7).

A trichloracetic acid extract from *Staphylococcus aureus* strain D was found to alter dermal reactivity of rabbits to epinephrine (8) in a fashion comparable to that of endotoxins from gram-negative bacteria and the lipoid A component of *E. coli* endotoxin(7). The same staphylococcal extract caused extensive hemorrhagic necrosis of mouse sarcoma 180(9). The present study was undertaken, therefore, to determine whether *E. coli* lipopolysaccharide and the corresponding lipoid A component also produce hemorrhagic necrosis of sarcoma 180 of mice, and to com-

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TABLE I. Comparative Necrotizing Effect of *E. coli* Lipopolysaccharide (LPS) and Lipoid A on Sarcoma 180.

7th day after tumor implantation		8th day after tumor implantation			
		Route of treatment			
		Intraper.		Intrav.	
Treatment			%		%
Extract	μg/mouse	No. of animals	positive	No. of animals	positive
None	0*	59	12	40	10
LPS	5	—	—	15	40
"	10	20	20	15	47
"	20	50	54	30	70
"	40	20	70	20	95
Lipoid A	25	—	—	15	40
"	50	10	50	25	64
"	100	30	70	19	68
"	200	10	90	10	100

* Control animals were inj. with equivalent amounts of saline solution.

pare the effects of the *E. coli* lipopolysaccharide with that of the same staphylococcal extract.

Materials and methods. Sarcoma 180 was implanted subcutaneously in female Ha ICR Swiss mice weighing between 18 and 23 g. The trocar transplantation technic and caliper measurements of tumor diameters were previously described(10). Lipoid A was prepared from the highly purified lipopolysaccharide obtained from *E. coli* 0111:B4 by hydrolysis and fractionation with organic solvents(6). This preparation, analyzed at the Freiburg laboratory, was composed of D-glucosamine, phosphoric acid (ester), and the 4 long-chain fatty acids, lauric, myristic, palmitic, and beta-hydroxy-myristic acid. It contained less than 0.01% of polysaccharide. The lipoid A material was dissolved in 0.5% dextran of low molecular weight. The lipopolysaccharide and lipoid A preparations used were the same as those used in the previous study(7). Each preparation was injected either intraperitoneally or intravenously into tumor bearing mice on the 7th day after implantation; control animals received saline. At time of treatment the average diameter of the tumors ranged from 11 to 13 mm. The effects were observed on the following day by gross inspection of the tumor and of the overlying skin, both in the intact animal and after exposure of the tumor, as described previously(9). Histologic examination of the lesions of several animals confirmed the gross findings in each case.

Results. The data on the tumor-necrotizing efficacy of *E. coli* lipopolysaccharide and its corresponding lipoid A fraction are shown in Table I.

Lipoid A, like the original lipopolysaccharide, produces hemorrhagic necrosis in 90 to 100% of the tumors. Approximately 4 to 5 times larger amounts of lipoid A than of the lipopolysaccharide were required to obtain the effect in a similar percentage of animals. Intravenous administration of lipopolysaccharide produced hemorrhagic necrosis in a larger percentage of mice than intraperitoneal injection; lipoid A was equally effective when given by these 2 routes. From these results it is concluded that the polysaccharide-free lipoid A component of *E. coli* 0111:B4 endotoxin produces hemorrhagic necrosis in sarcoma 180, similar to the original lipopolysaccharide, but that it is less effective on a weight basis.

The results of the evaluation of the *E. coli* lipopolysaccharide and of the staphylococcal extract are summarized in Table II. Both bacterial preparations caused tumor necrosis at doses as low as 10 μg per mouse; the staphylococcal extract, however, was slightly more potent than the *E. coli* lipopolysaccharide. Different rates of absorption of the 2 preparations from the peritoneal cavity may be responsible for this difference, for in one experiment both extracts were equally active when administered intravenously.

TABLE II. Comparative Necrotizing Effect of *E. coli* Lipopolysaccharide and Staphylococcal Extract on Sarcoma 180.

Treatment		Hemorrhagic necrosis	
Extract	μg/mouse	No./Treated	% positive
None	0*	6/50	12
E. coli lipopolysaccharide	10	2/10	20
	20	23/40	58
	40	7/10	70
S. aureus D extract	10	6/10	60
	20	18/20	90
	40	9/10	90

* Control animals were given equivalent amounts of saline solution.

Discussion. Endotoxins of gram-negative bacteria have attracted renewed interest during the past few years. The extraordinary number of biologic effects that they exert in susceptible animals have prompted further studies on their chemical composition in relation to their biologic activity. The lipopolysaccharide moiety exerts most, if not all, of the biologic effects. The lipoid A component of this complex was found to be pyrogenic, to alter nonspecific resistance to experimental infection, and to enhance reactivity of the rabbit to epinephrine(6,7). The present study describes an additional activity of the lipoid A component, namely, that this fraction can cause hemorrhagic necrosis of mouse sarcoma 180. In the present experiments, lipoid A was approximately 5 times less effective than the lipopolysaccharide complex from which it was derived. The tumor necrotizing activity of lipoid A cannot be attributed to contamination with intact lipopolysaccharide, for careful examination for sugar constituents showed that the lipoid A preparation used contained less than 0.01% of polysaccharide. It is conceivable that only a portion of lipoid A is responsible for the biologic effect described here. It is important to point out in this connection that by aqueous ether extraction Ribi and associates obtained highly effective endotoxic products with a low lipoid content(11). Products containing 1 to 4% bound lipoid were as toxic as endotoxins containing as much as 15 to 30%. The identity of the chemical determinant(s) responsible for the numerous biologic activities of endotoxins requires further study.

Recently, Schaedler and Dubos(12) observed that albino mice (Rockefeller NCS strain) raised and maintained free of ordinary bacterial pathogens, including *Escherichia coli,* were highly resistant to the lethal effect of bacterial endotoxin, but highly susceptible to its infection-enhancing effect. With the acquisition of intestinal flora from other mice the animals became susceptible to the lethal effect of endotoxin. In view of these observations the mechanism of the tumor necrotizing effect of endotoxins (lipopolysaccharides) and of their subfractions, such as lipoid A, deserves further investigation.

Most of the previous investigations on endotoxins were carried out with products obtained from gram-negative bacteria. Recently, a trichloracetic acid extract of *S. aureus* was found to alter dermal reactivity of the rabbit to epinephrine(8), in a fashion comparable to that of *E. coli* lipopolysaccharide and its lipoid A component(7). The same staphylococcal extract also causes hemorrhagic necrosis of mouse sarcoma 180(9, 13). In addition, Higginbotham and Bass found that the Fritchie strain of *S. aureus* has the same activity on this tumor(13). In the present study, the tumor necrotizing efficacy of the staphylococcal extract of strain D was of the same order of magnitude as that of the *E. coli* lipopolysaccharide. Further investigations are necessary to determine the composition of the toxic factor(s) of staphylococci. Definition of this factor as endotoxin must await clarification of the chemical relationship, if any, between the staphylococcal toxin and the endotoxins of gram-negative bacteria.

Summary. The polysaccharide-free lipoid A component of *E. coli* 0111:B4 endotoxin produces hemorrhagic necrosis in sarcoma 180 of mice, but is somewhat less effective than the lipopolysaccharide from which it was derived. A trichloracetic acid extract obtained from *S. aureus* strain D was slightly more effective than the *E. coli* lipopolysaccharide on this tumor.

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Detoxification of Bacterial Endotoxin with Retention of Ability to Stimulate Non-Specific Resistance to Infection. (26766)

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The highly pyrogenic and toxic endotoxins elicit, at microgram doses, a wide spectrum of biological effects including stimulation of non-specific host resistance to infection(1-3). Attempts at dissociation of *in vivo* properties of endotoxin by chemical modification, largely by hydrolytic measures, have been unsuccessful. This preliminary report of our studies on chemical modification of endotoxin, utilizing reactions resulting in substitution at functional groups known to be present in the molecule, describes a fraction isolated after acylation which is grossly detoxified but retains quantitatively the ability to stimulate resistance to infection with heterologous microorganisms. Recovery of original pyrogenicity and acute toxicity by mild saponification, failure to confer tolerance to the original endotoxin, degree of immunogenicity, retention of tumor-necrotizing activity, as well as the properties of other fractions isolated, will be reported separately. While our manuscripts were in preparation Noll and Braude(4) reported detoxification of endotoxin by reductive cleavage of ester bonds, yielding a product of high immunogenic potency.

Materials and methods. In all procedures, preparative and testing, usual precautions to avoid pyrogen contamination were observed. Preparation was accomplished by adding 25 ml acetic anhydride to 100 mg *S. typhosa* 0-901 endotoxin (Difco) and 10 mg anhydrous sodium acetate, and heating in a boiling water bath for 2 hours. After cooling, the anhydride was removed as a clear solution containing approximately 30% of the starting material, by centrifugation. The residue was dried under N_2 to remove all anhydride, washed 3 times with pyrogen-free saline and finally with water, the washes removing a fraction (20%) yielding a fine suspension like the original endotoxin. The washed residue (50% yield), which is the subject of this report, was dried under N_2 and ground to a fine powder in a mortar. This lyophobic fraction was triturated with small amounts of pyrogen-free saline, then diluted to desired concentrations. Acetyl content, by alkaline hydrolysis, was 12% compared to 1.6% for the original endotoxin.

Tests for acute toxicity and protection against infection were done with ICR mice (Bellewood) weighing 16-18 g. For pyrogen tests 2-2.5 kg male rabbits of mixed breed were used. Dosage, routes of administration, and time schedules are given below. Strains of *Pseudomonas aeruginosa* and *Escherichia coli* from our culture collection were employed in the resistance studies described below. Both organisms were grown in brain heart infusion broth (Difco) for 18 hours at 35°C and appropriately diluted in sterile broth prior to injection of 0.25 ml *i.p.* The *E. coli* inoculum consisted of approximately 2×10^8 viable cells per ml while the *Ps. aeruginosa* viable count amounted to about 6×10^8 cells per ml.

Results. The data in Table I demonstrate the reduction in acute toxicity: the acetyl-

TABLE I. Acute Toxicity of Endotoxin and Acetylated Fraction in Mice.

Dose, mg	Dead/Total	
	Endotoxin	Acetylated fraction
.3	13/20	1/10
.6	17/24	0/9
.9	10/10	0/10

All doses given *i.p.* in saline with deaths recorded for 72 hr after inj.

ated fraction was non-lethal at a dose which for the original endotoxin killed all animals. Another preparation, available in larger amount, was also non-lethal at 1 mg but produced deaths at 2 mg. Reduction in pyrogenicity is illustrated in Fig. 1. The data in-

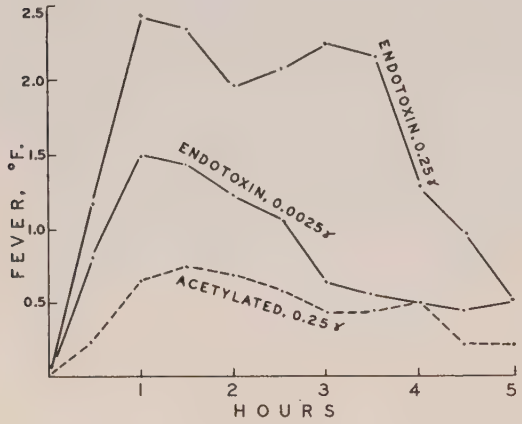

FIG. 1. Mean febrile responses of groups of 6-8 rabbits each, given original endotoxin or acetylated fraction *i.v.*

dicate a reduction by one-thousand fold; individual preparations, by this method, have ranged from 1/100 to 1/10,000 the original pyrogenicity.

The resistance to a lethal challenge of *Ps. aeruginosa* or *E. coli* in mice pretreated with endotoxin or the acetylated fraction was significantly increased compared to the saline controls (Table II). In this system the degree of resistance elicited by microgram doses of either endotoxin or its detoxified derivative was essentially the same.

Discussion. The acetylated fraction of bacterial endotoxin described herein represents a clear dissociation of *in vivo* properties, the toxic and pyrogenic effects being grossly reduced without alteration of stimulation of non-specific resistance to infection. The latter, it should be emphasized, is demonstrated with doses of the same order of magnitude as those shown for determining pyrogenicity. Additional data also indicate equivalence between the 2 materials in promoting resistance to infection with various other gram-negative and gram-positive microorganisms. That this selective reduction in toxic properties is attributable to the acetyl groups introduced is supported by the finding, to be reported, that the original pyrogenicity and acute toxicity are recovered by de-acetylation with alkali. The detoxified immunogenic preparation recently reported by Noll and Braude(4) confers tolerance to both the pyrogenicity and lethality of the original endotoxin; it is of interest that the acetylated fraction described above does not confer such tolerance, measured by the same parameters.

Summary. The dissociation of biological properties of bacterial endotoxin by chemical modification is described. A fraction iso-

TABLE II. Increased Resistance to Infection in Mice Following Administration of Endotoxin and Acetylated Fraction.

Group	Dose, μg*	Challenge organism	Dead/Total†	% survivors
Saline	—	*Ps. aeruginosa*	10/10	0
Endotoxin	1	" "	6/10	40
"	10	" "	4/10	60
Acetylated fraction	1	" "	6/10	40
" "	10	" "	3/10	70
Saline	—	*E. coli*	7/10	30
Endotoxin	0.1	" "	4/10	60
"	1	" "	2/10	80
Acetylated fraction	0.1	" "	4/10	60
" "	1	" "	2/10	80

* All doses given *i.p.* in 1.0 ml of saline 24 hr prior to challenging infection.
† Deaths recorded after 72 hr.

lated after acetylation of endotoxin exhibits grossly reduced pyrogenicity and acute toxicity but retains the original potency in stimulating non-specific host resistance to infection.

The authors are indebted to Prof. A. S. Gordon for suggesting we undertake studies on chemical modification of endotoxin, and for his encouragement and criticism. The technical assistance of Miriam Graff and Romus Broadway is gratefully acknowledged.

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Suppression of the Foreign Bone Marrow Reaction by Preirradiation of Donor Mice. (26767)

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The bone marrow of normal mice is composed of a mixed population of cells differing in morphology and function. On transplantation of viable marrow cells into lethally irradiated homologous recipients, various blood-forming elements repopulate the marrow, the red pulp of the spleen [see review article of Smith and Congdon(1)] and the antibody-forming lymphoid tissues of the host(2,3). Probably only a small percentage of the marrow cells or their precursors are antibody-forming, since as few as 1×10^6 transplanted marrow cells protect against radiation lethality(4,5), whereas as many as 12×10^6 transplanted marrow cells do not confer on lethally irradiated recipients the capacity to synthesize antibodies(6). The radiation dose required to reduce cell survival by a factor of 0.37 is estimated at about 115 rads of Co^{60} rays for blood-forming cells(7) and 130 r of 250 kvp X-rays for spleen cells producing humoral antibodies(8). It seemed possible, therefore—the radiosensitivity of the 2 cell types being comparable—that the antibody-forming cells in mouse bone marrow could be largely eliminated by exposing the animal to an X-ray dose lethal to all but 2-6% of its total marrow population. Marrow thus deprived of immunologically active cells and transplanted into a lethally irradiated homologous recipient would presumably repopulate the recipient's hemopoietic sites but lack usual graft-versus-host immunological reactivity. Data presented here do, in fact, indicate that preirradiation of marrow donors reduces the incidence and severity of secondary disease in irradiated homologous recipients.

Materials and methods. Adult hybrid mice (5-8 months old) of the following strains were used as recipients and donors: (101/Cum × C3H/Anf Cum)F_1 (C57BL/Cum × 101/Cum)F_1, (C57L/Cum × A/He Cum)F_1, (C57BL/6 Cum × DBA/2 Cum)F_1, and (BALB/c Cum × A/He Cum)F_1. These strains are hereafter designated as $1C3F_1$, BlF_1, LAF_1, $B6D2F_1$, and CAF_1, respectively. In every case, donor and recipient were of the same sex. Recipients were exposed to 950 r whole-body X radiation; irradiated donors were exposed to 400 r or 500 r 30 minutes before sacrifice. In some instances, the donor mice were given a radioprotective compound, S, β-aminoethylisothiuronium · Br · HBr (AET), 20-30 minutes before irradiation. Nine milligrams of AET in 0.2 M phosphate buffer (pH = 7.4) were injected intraperitoneally per mouse. The X-ray dose rate was approximately 85 r/min; TSD, 93.5 cm; 300 kvp; 20 mA; 4.78 mm of Be inherent filtration and 3 mm of Al added filtration, and hvl, 0.55 mm of Cu.

* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

TABLE I. Survival of Lethally Irradiated Mice Following Implantation of Homologous Marrow (30 Mice per Group).

Recipient strain	Donor		No. surviving 21 days	No. surviving 90 days		Incidence secondary morbidity and mortality (%)
	Strain	Treatment		Secondary disease present	Secondary disease absent	
$B1F_1$	CAF_1	None	29	0	0	100
		400 r	22	6	9	59
$1C3F_1$	$B6D2F_1$	None	30	2	0	100
		400 r	28	4	13	53
$1C3F_1$	LAF_1	None*	27	0	0	100
		"	30	6	0	100
		AET	30	3	0	100
		400 r	24	0	15	37
		AET + 400 r	30	6	18	40
		500 r	20	2	18	10
		AET + 500 r	28	0	28	0

* 1.5×10^6 nucleated cells inj. instead of 60×10^6.

Marrow cells were flushed from the femur with Tyrode's solution using a syringe with a 24-gauge needle. The nucleated cells were counted in a hemocytometer. Recipients were inoculated in a tail vein with 60×10^6 nucleated cells within 3 hours after irradiation, unless otherwise stated. The hemoglobin type in the peripheral blood of marrow recipients was characterized(9) at 60 and 90 days after marrow infusion to ascertain whether the donor cells had transplanted. Mortality was recorded for 90 days after treatment; the terms "early death" and "delayed death" refer to death during the first 21 days and during the 21- to 90-day-period after irradiation, respectively. The incidence of secondary disease was estimated by including the dead and clinically affected mice surviving more than 21 days after irradiation.

Results. The experimental results are presented in Table I. In all host-donor combinations, the unirradiated marrow protected against early radiation lethality. Secondary mortality, however, occurred during the second and third months. Variations in severity and incidence of secondary disease were observed in different host-donor strain combinations, but not in relation to number of marrow cells transferred. Preirradiation of donor mice with 400-500 r usually reduced the ability of 60×10^6 nucleated marrow cells to protect irradiated recipients against early radiation lethality; however, such animals surviving 21 days exhibited symptoms of secondary disease less frequently than comparable recipients of unirradiated marrow. Secondary disease, recognized by weight loss, diarrhea, and delayed hair-graying was almost invariably fatal to mice protected with unirradiated marrow. However, the disease was mild and transient in recipients of irradiated marrow. The latter mice survived and their hair grayed 40 to 90 days after irradiation. When LAF_1 donor mice were given AET before exposure to 400-500 r, the marrow was just as effective in preventing early radiation death as marrow from unirradiated donors, indicating that a significant number of hemopoietic donor cells were protected against radiation damage by the AET. In the recipients given marrow from irradiated donors either treated with AET or not, incidence and severity of secondary disease was equally low, which suggests that the compound did not protect antibody-forming cells in the marrow. Chemical treatment of unirradiated donors had no detectable effect on the occurrence of secondary disease. All the $1C3F_1$ recipients of LAF_1 or $B6D2F_1$ marrow that survived more than 90 days after treatment, had donor-type hemoglobin (90-100% of total hemoglobin) in the peripheral blood, irrespective of the treatment given the donor. All the $B1F_1$ recipients of irradiated CAF_1 marrow had donor-type hemoglobin (90-100% of total) 60 days after treatment,

but by 90 days the hemoglobin had reverted to host type in 10 out of 15 mice. Probably the number of irradiated viable CAF_1 blood-forming cells inoculated was suboptimal, since the CAF_1 mouse is more radiosensitive than the other 2 donor strains employed(10). Regression of the grafted marrow in homologous chimeras occurs when small numbers of donor cells are inoculated into the irradiated recipients(11).

Discussion. The incidence and severity of secondary disease in homologous bone marrow chimeras was reduced by pre-exposure of the donor mice to 400-500 r of total-body X radiation, which strongly indicates that immunologically competent cells or their precursors in the donor marrow did not survive irradiation in large numbers. Conversely, 90-100% of the hemoglobin in the blood of the chimeras was of donor type, indicating that erythropoietic cells of the irradiated marrow survived in large enough numbers to repopulate the host, although 400-500 r were estimated to have killed 94-98% of all donor hemopoietic cells(7). Transferring a small number of unirradiated bone marrow cells, the number being comparable to that estimated to survive 400 r, does not prevent secondary disease in homologous chimeras. Hence, a nonselective reduction of the overall population *per se* does not reduce the incidence of secondary disease and is not the mechanism by which preirradiation of the marrow donors suppressed secondary disease in homologous chimeras.

Simonsen(12) and Popp(13) suggested that immunologically competent cells transplanted at different stages of maturation may behave differently in homologous recipients, the more mature cells reacting against host-type transplantation antigens, the immature ones being unresponsive when they mature. It may be postulated, therefore, that irradiation of donor mice eliminated mainly the mature antibody-forming cells and that the immature ones surviving may have become specifically unresponsive to the host antigens. Preliminary tests, *i.e.*, retransplantation of chimeric spleen cells, indicate that the immune machinery in the surviving recipients of irradiated marrow is of donor type (Cudkowicz, unpublished data).

Administration of a radioprotective compound to the donor mice to be irradiated apparently had a selective effect in that the hemopoietic cells survived irradiation treatment better than the cells reacting immunologically against transplantation antigens. This conclusion is inferred from the fact that donor hemopoietic cells were functional at time of transplantation and were able to proliferate and to repopulate the host. Nevertheless, the chimeras failed to develop immunological disorders (secondary disease), implying that the irradiated immunologically competent cells were not protected by AET. That AET was able to protect hemopoietic elements of marrow against radiation injury agrees with results obtained by Smith(14) and by Doherty(15). That the drug *did not protect* immunologically competent cells against radiation injury to the same extent is supported by other direct(16) and indirect evidence(17).

Summary. Preirradiation of donor mice with 400-500 r of X-rays resulted in a marked reduction of secondary disease in homologous radiation bone marrow chimeras. The results are tentatively attributed to elimination of immunologically competent cells from donor marrow by X radiation to an extent adequate to inhibit the development of clinically significant graft-versus-host immune reactions. This effect was not detectably inhibited by administration of a radioprotective compound (AET) 20-30 minutes before irradiation of donor mice, implying that AET did not protect immunologically competent cells to the same extent as hemopoietic cells against radiation injury.

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Modification of the Friend Virus Disease by Splenectomy.* (26768)

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Friend Virus infection in Ha/ICR Swiss mice produces a syndrome characterized by 2 distinct responses. The first, a malignant disorder, involves uncontrolled proliferation of reticulum cells with massive involvement of spleen and liver. The second response involves the hematopoietic system and manifests itself by an increased production of erythroblasts, erythrocytes and leukocytes. Previous studies by Mirand, *et al.*(1) demonstrated selective uptake of Fe^{59} in the spleen of infected animals and supported the hypothesis that the increased erythropoiesis was largely due to increased erythropoietic activity in the spleen. Further studies of the relationship of the spleen to the hematopoietic response were done and constitute the basis of this report.

Method. Mice employed in this study were Ha/ICR Swiss male mice 6-8 weeks old, obtained from our own breeding colonies and maintained on a diet of Derwood-Morris pellets.

Friend Virus(2) was obtained through the courtesy of Dr. Charlotte Friend, Sloan-Kettering Institute, New York City and was in its 12th serial passage in our laboratory at the beginning of this study. A pool of the virus was obtained from this passage by homogenization of 10% infected spleen mince suspension in cold Locke-Ringer solution, clarification by centrifugation for 10 minutes at 5000 G at 5°C and subsequent filtration of the supernate through a HA-grade Millipore filter with an *E. coli* marker. This filtrate was stored in sealed ampoules in a dry ice chest until used. The standard inoculum for each mouse was 0.2 ml intraperitoneally. This inoculum produced typical disease in about 85-90% of our mice. A minimum of 15 mice was used for each point in the various experimental groups.

Hematocrits were determined with Drummond microhematocrit capillary tubes. Leukocyte and erythrocyte counts were done in standard human diluting pipettes and a hemocytometer. Blood films were stained with Wright-Giemsa stain.

In the groups having splenectomies, the procedure was performed 3 days prior and 4, 19, 21 and 27 days after inoculation of the virus.

Results. Table I illustrates the characteristic splenomegaly which occurs in Ha/ICR Swiss mice following infection with Friend Virus. The rapidity with which splenic enlargement occurs is related largely to the size of the original inoculum. When a dilution of 10^{-1} was inoculated there was roughly

* This study was supported in part by grants from U. S. Public Health Service, John A. Hartford Foundation, Inc. and Atomic Energy Commission.

TABLE I. Change of Spleen Weight in Ha/ICR Swiss Mice Inoculated with Friend Virus of Dilution Varying from 10^{-1} to 10^{-5}. Spleen weights in g as function of time after inoculation.

Days	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
0	.14	.15	.15	.14	.15
6	1.6	1.51	.17	.14	.15
12	2.0	1.12	.32	.18	.15
18	1.8	1.7	1.1	.16	.28
24	2.6	2.0	1.5	.34	.20
30	3.1	2.3	1.6	.58	.22
36	2.9	2.25	2.1	1.32	.18
42	2.2	2.1	2.2	2.2	.20

a 10-fold increase in splenic weight in 6 days (Table I). With higher dilutions more time was required for development of splenomegaly. While the increase in spleen weight occurred rapidly in the early post-infection period it soon reached a plateau (time depending on titer of inoculum) and remained relatively constant thereafter.

The leukocytosis which accompanied the splenomegaly is shown in Fig. 1 and Table II. The upper curve in Fig. 1 represents total circulating leukocyte count and Table II shows the relative numbers of granulocytes and lymphocytes. The level of leukocytes in the peripheral blood rose rapidly and reached a peak at about 20 days. Thereafter, there was a gradual decline which leveled off at lower but still significantly elevated levels. Microscopic study (Table III) of the white cells in the peripheral blood revealed essentially only immature and mature leukocytes without definitely abnormal forms of either lymphocytes or granulocytes. This might be aptly termed a "leukemoid reaction" such as may occur in response to an infectious process. Table III shows a marked granulocytosis that usually appears 26 days after infection.

One of the most sensitive indicators of Friend Virus infection was previously shown to be the development of a peripheral erythroblastosis(1,2,3). This response is shown in Table II in the line marked controls under erythroblasts.

In splenectomized mice, the marked leukocytosis following infection with Friend Virus did not occur. Table II shows that the leukocyte count was not significantly elevated as long as 48 days post-inoculation. These mice were splenectomized 3 days prior to inoculation with a 10^{-1} dilution of the virus. The controls were animals which received the same inoculum of virus but were not splenectomized. Fig. 1 shows a comparison of leukocyte levels between mice splenectomized 3 days prior to virus inoculation and mice splenectomized 4 days after virus infection. The upper curve in Fig. 1 represents the non-splenectomized controls receiving the virus and the lower lines represent splenectomized mice which were followed for a total of 60 days. No peripheral leukocytosis appeared in the splenectomized group observed for 2 months.

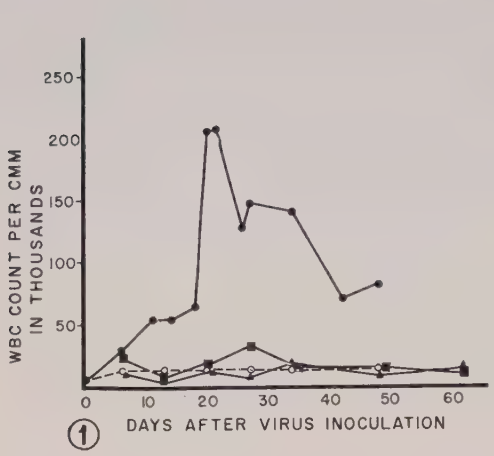
Fig. 2 and 3 show comparable data for the hematocrit and red blood count observed in the non-splenectomized controls whereas no rise was observed in splenectomized mice during this period. Likewise, the usual appearance of large numbers of erythroblasts in the

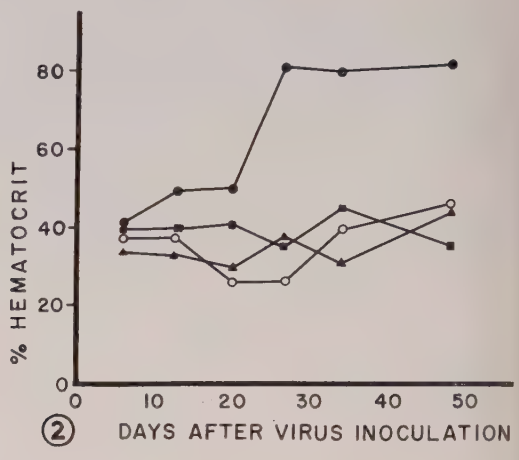
TABLE II. Effect of Splenectomy on Ha/ICR Swiss Mice Response to Friend Virus as Measured by Circulating Blood Cell Count. Mice splenectomized 3 days before virus inoculation. Cell counts in 10^3 per mm^3.

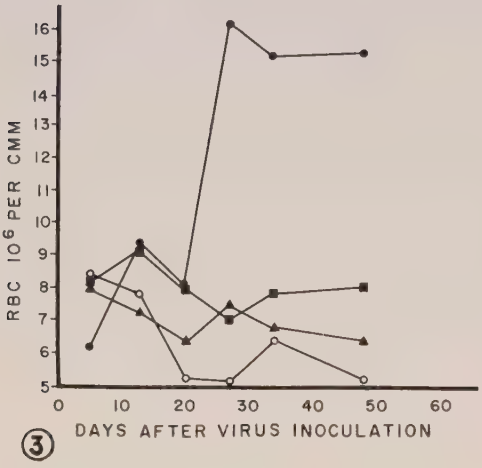
Days after virus inoc.	6	13	20	27	34	48
Granulocytes	5.5	1.2	4.7	2.2	8.2	3.9
Controls*	9	13	100	62	53	40
Lymphocytes	7.7	3.4	6.3	5.7	10	4.6
Controls*	17	25	115	72	50	40
Erythroblasts	0	0	0	.01	.02	0
Controls*	0	1.4	4.0	28	12	11
Liver wt (g)†	2.42	2.34	2.50	2.00	2.46	2.68

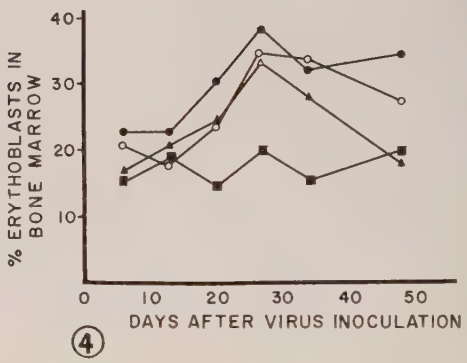
* Controls are cell counts in mice not splenectomized but which were inoculated with 10^{-1} of standard virus filtrate.

† Liver weights in mice splenectomized 3 days before virus inoculation. Avg normal liver wt is 2.40 g.









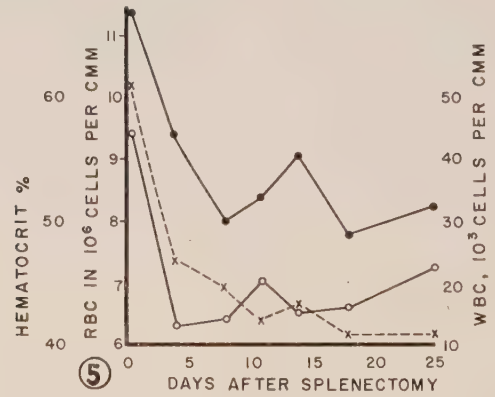


FIG. 1. Circulating total WBC count in Friend virus infected Swiss mice: —●—●—, infected non-splenectomized mice; –○– – –○–, splenectomized mice not virus infected; ▲, mice splenectomized 3 days prior to virus inoculation; ■, mice splenectomized 4 days after virus infection.

FIG. 2. Hematocrit expressed as % in Swiss mice inoculated with Friend virus plotted against time after inoculation. ●, non-splenectomized infected mice. ▲, mice splenectomized 3 days before inoculation. ○, mice splenectomized 4 days after inoculation. ■, control mice splenectomized but without virus inoculation. All inoculations at 10^{-1} standard filtrate.

TABLE III. Representative Counts of Blood of Ha/ICR Swiss Mice* Receiving Friend Virus (10^{-1}).

Days	HT	Total WBC count	Neutrophils M	J	ST	Seg	Lymphocytes Blast	Ma-ture	Mono-cytes	Other abnormal cells	Wt Spleen	Wt Liver
0	42	7,550			1	26	2	69	2	1 Ferrata	.10	2.10
6	39	19,400			4	26	1	68	1		.76	2.39
13	47	34,850		1	3	33	5	50	5	2 Megakarocytes	1.13	2.46
20	52	109,000	1	3	7	36	1	44	8	3 Friend, 1 Rieder	1.70	2.87
26	81	60,000	4	5	12	26	7	40	6	2 Friend	3.60	2.79
34	74	70,070	2	4	9	46	5	31	3	4 Friend	2.12	2.83
48	83	46,000	3	8	11	36	5	29	8	2 Ferrata, 1 Rieder	1.66	2.89
62	70	274,000	2	4	10	59	3	19	3	3 Friend	2.46	2.73

* Non-splenectomized Swiss mice.

peripheral blood did not occur in the splenectomized group. It is interesting, however, that examination of the bone marrow of the infected splenectomized group showed some increase in numbers of erythroblasts in the bone marrow (Fig. 4). However, these did not appear in the peripheral blood and there was no increase in peripheral erythrocyte levels.

This interesting relationship of the spleen to development of leukocytosis, erythroblastosis and erythrocytosis prompted us to study the effect of splenectomy in mice which had already developed the full blown Friend Virus Disease syndrome. Mice were inoculated with a 10^{-1} dilution of virus and were divided into 3 groups. The first group was splenectomized 19 days post-inoculation, the second group at 21 days and the third at 27 days. Portions of each group were kept as non-splenectomized controls. Prior to splenectomy, hematocrits, leukocyte and erythrocyte counts were done. Since the same results were seen in the 19 and 27 day groups, Fig. 5 shows data only on mice which were splenectomized 21 days post-infection. There was a striking drop of the elevated hematocrit, erythrocyte and leukocyte levels. The infected non-splenectomized animals maintained the usual increased levels of these parameters.

Discussion. These results indicate that the phase of Friend Virus Disease which is manifested by leukocytosis, increased hematocrit, erythroblastosis and erythrocytosis (hematopoietic response) is intimately related to the spleen. Not only did pre-infection splenectomy prevent this response, but splenectomy of animals with early or already fully established disease, abolished it. In infected splenectomized mice the only suggestion of the hematopoietic response was an increase in erythroblasts in the bone marrow. However, there was no peripheral erythroblastosis and no elevation of hematocrit or erythrocyte levels. Also it was previously demonstrated that no significant increase of Fe^{59} uptake occurred in the bone marrow(1). At no time did the bone marrow in any infected group (control or splenectomized) show evidence of increased activity of the myeloid elements.

The mechanism of splenic control of the hematopoietic response in the Friend Disease is not clear. However, these and previous studies(1) indicate that the hematopoietic

FIG. 3. Count of circulating red blood cells in splenectomized Swiss mice with Friend virus. Legend same as for Fig. 2.

FIG. 4. Percent erythroblasts in bone marrow of Friend virus infected Swiss mice. –●—●–, infected non-splenectomized mice; ■, splenectomized controls without virus inoculation; ▲, mice splenectomized 3 days before virus inoculation; ○, mice splenectomized 4 days after virus inoculation.

FIG. 5. Effect of splenectomy on circulating blood elements in Friend virus infected Swiss mice; virus was inoculated 21 days prior to splenectomy. ●, RBC count; –×––×–, hematocrit; ○, total WBC count.

elements in the spleen are primarily responsible for production of the increased numbers of immature and mature erythrocytes and leukocytes. This is certainly true for the erythropoietic picture and for the leukocytic picture up to 48 days after post-infection. Since at this time the liver and particularly the bone marrow show no significant increase in activity in either splenectomized or non-splenectomized infected mice, the stimulus to hematopoietic activity seems to be specific for those elements in the spleen. One hypothesis is that the virus infects the reticulum (or other target) cells of the spleen and then, by some mechanism, stimulates secondarily the hematopoietic elements of that organ. It is puzzling that the same phenomenon would not occur to the same degree in the bone marrow and liver. However, it may be that the target cells in the spleen are in some way different from morphologically similar cells in the bone marrow and liver, thus accounting for the splenic specificity. If the increased hematopoietic activity were due to a direct action of the virus on the blood forming elements *per se*, one might expect that the bone marrow elements would be similarly stimulated unless again, there were differences among the splenic, liver and bone marrow hematopoietic elements. We feel that the first possibility is a more likely explanation but additional studies are needed before any conclusions are warranted.

Although we believe that the spleen is primarily responsible for the erythropoietic and leukocytic response herein reported, there is some evidence to believe that the liver plays a prominent role in the leukocytosis of splenectomized mice after 48 days post-infection. Note in Fig. 5 that the hematocrit, erythrocyte and leukocyte levels declined progressively from high levels after splenectomy, but at 25 days post-splenectomy or 46 days after inoculation of the virus the leukocytes were reduced but again elevated to 22,000 mm^3. At 55 days post-splenectomy or 76 days after inoculation of the virus (not shown on Fig. 5) the hematocrit dropped to 22%, red blood count to 3.1 million and the white blood count increased to 282,000 mm^3. This increase in white blood count paralleled the development of hepatomegaly. Histological examination of the livers in this group demonstrated at this time uncontrolled proliferation of reticulum cells, few erythrocytic foci and many foci of mature cells of the granulocytic series. There was no evidence of bone marrow hyperplasia and in many instances the bone marrow appeared depressed. It was apparent in this group of mice that splenectomy does prevent the erythropoietic response in the disease but does not prevent eventual development of the malignant reticulum cell leukemia and only delays the appearance of leukocytosis until hepatomegaly occurs.

It is important to stress here that although splenectomy modified the hemapoietic response in the Friend Virus Disease, it did not alter the eventual development of reticulum cell leukemia. Gross(4) has shown, however, that thymectomy either before or after inoculation of "Passage A" leukemic filtrates inhibited viral induction of lymphocytic leukemia in C_3H mice.

Summary. The Friend Virus syndrome in Ha/ICR Swiss is fundamentally modified by splenectomy either 3 days before or 4, 19, 21 and 27 days after inoculation of the virus. Peripheral erythroblastosis and erythrocytosis only occur in presence of the spleen. The characteristic leukocytosis is not seen up to 48 days after infection in splenectomized mice; however, leukocytosis appears again after gross hepatomegaly. Although the hemopoietic syndrome of the Friend Virus Disease is modified by splenectomy, the virus induced reticulum cell leukemia eventually appears.

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A Type 3 Attenuated Poliovirus Genetically Stable after Human Intestinal Passage.* (26769)

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The type 3 strains of attenuated poliovirus available fail to maintain their genetic markers after passage through the human intestinal tract(1-5). Although no epidemiological evidence has been adduced so far for reversion of these strains to agents virulent for man(6,7), the fact that *in vitro* markers may change to those frequently indistinguishable from virulent virus makes it more difficult to monitor the live virus vaccination program. As changes in genetic character of type 3 strains are occasionally accompanied by an increase in pathogenicity for monkeys, public health authorities have expressed concern for the safety of contacts of subjects fed the live attenuated type 3 strains presently available (3,8).

The reproductive capacity of type 3 viruses isolated from feces of vaccinated subjects in tissue culture systems incubated at 40°C (rct/40°C marker)† has been used most frequently as a test of vaccine strain stability, since a fair correlation has been established between the rct/40°C marker and other markers, including pathogenicity for monkeys(3). In Tables I and II, respectively, are summarized the rct/40°C characteristics of virus strains representing the first human intestinal passage of the 2 attenuated type 3 strains that have been used most widely in mass immunization programs: Leon KP-34(9) and W-Fox(10). Both strains grow relatively poorly in tissue culture systems kept at 40°C and are therefore classified as rct/40°C–; however, when fed to man, both strains tend to induce excretion of viruses which were found to replicate equally well at 37°C and 40°C and are therefore classified as rct/40°C+.

As shown in Table II, results of 4 studies of the Leon KP-34 indicated that a majority of subjects fed this strain excreted rct/40°C+ virus. Similarly, it has been found that children fed W-Fox strain (Table I), in 16 out of 16 cases excreted strains which were rct/40°C+.

Because of the instability of these viruses, an attempt was made to develop an attenuated type 3 strain which would remain genetically unaltered after passage through the human intestinal tract.

Materials and methods. Tissue culture systems. Freshly harvested explants of Rhesus monkey kidney tissue were used throughout the study. Cultures were prepared from either one million kidney cells dispersed in petri dishes or 200,000 cells in culture tubes which had been incubated at 37°C in growth medium consisting of Eagle's medium in Earle's solution with calf serum to a 10% concentration. After 6 days' incubation in presence of 4% CO_2 in air inflow, the growth medium was replaced by maintenance medium consisting of Eagle's medium in Earle's solution without serum. At the same time the cultures were exposed to type 3 virus preparations. Cultures in tubes were used for virus passage purposes and for titration of virus preparations at different temperatures of incubation, whereas plates were used for cloning the virus at different passage levels and for titration of stools at several temperatures. For passage in tube culture, 3 tissue culture tubes were exposed to 0.1 ml of undiluted virus from the previous passage. After one hour's incubation at room temperature (25°C) the virus was removed, the tubes washed once, and fresh maintenance medium added. The tubes were incubated at 23 or 37°C until 50% cellular degeneration was noted, or until cells in the non-infected control tubes began to degenerate. This latter period varied between 12 and 16 days at the

* This work was supported in part by grant E. 1799 from U.S.P.H.S.

† Reproductive capacity at temperature 40°C. Determined by dual titration of a virus at 40°C in comparison with control titration at 37°C.

TABLE I. Reproductive Capacity at 37°C and 40°C of Fecal Virus Isolates from Infants Either Fed $10^{5.5}$ TCD_{50} of the W-Fox Strain or Infected by Contact.

Subject	No. days post-vacc. virus isolated	Material tested	$Log_{10}TCD_{50}$ 37°C	$Log_{10}TCD_{50}$ 40°C	$Log_{10}\frac{TCD_{50}37°C}{TCD_{50}40°C}$*
W-Fox	—	Vaccine	7.7	4.7	3.0
H-24	—	Virulent	8.2	8.0	.2
B-157	8	St	4.4	3.7	.7
I-1	11	"	2.4	1.9	.5
I-1	7	1TC	7.5	5.7	1.8
I-6	5	St	1.9	1.4	.5
I-6	5	1TC	7.5	7.2	.3
Q-1	10	"	7.7	7.7	.0
L-1	9	"	6.7	7.2	−.5
R-1	7	"	7.5	7.2	.3
WB-110	7	St	3.9	2.2	1.7
Q-5	18	1TC	6.2	5.7	.5
I-4	9	"	7.5	7.2	.3
I-5	4	"	7.2	7.5	−.3
I-7	2	"	6.5	6.7	−.2
R-4	18	"	6.7	5.7	1.0
L-5	7	"	7.2	6.5	.7
E-5	10	"	7.7	7.2	.5
E-4	10	"	7.2	7.2	.0
B-158	5	St	3.5	2.9	.4
B-158	5	1TC	7.7	7.5	.2

* Controls: W-Fox, 3.3; H-24 (virulent), 0.2.

St = Stool; 1TC = First tissue culture passage.

TABLE II. Reproductive Capacity at 40°C (ret/40°C marker) of Type 3 Leon KP-34 Attenuated Strain of Sabin after Human Intestinal Passage.

Laboratory	Properties of fecal virus. No. of specimens Tested	ret/40°C+	ret/40°C±	ret/40°C−
Sabin(1)	42	15	20	7
	27*	26	1	0
Gendon *et al.*(2)	17	5	4	8
Melnick & Benyesh-Melnick(3)	23	12	2	9
Verlinde & Wilterdink(4)	13	6	3	4
Total	122	64	30	28

* Viruses isolated from contacts.

23°C temperature of incubation. The contents of the tubes were then rapidly frozen and thawed 3 times and pooled: this material was then used as seed virus for the next tissue culture passage.

In the case of the plate cultures the virus was adsorbed for one hour, the excess removed by washing, and the monolayers covered with 5 cc of an agar overlay maintenance medium consisting of equal volumes of 2% Noble's agar and of a nutrient solution (11). On the second day post-inoculation the plates were covered with a second overlay containing neutral red and the appearance of plaques determined on the 3rd to 7th day of incubation.

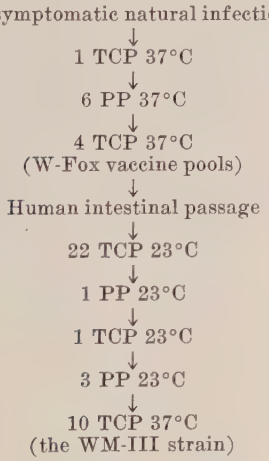
Plaque passage was performed by exposing kidney monolayers to virus at terminal dilution, and by selection of one of the plaques at random, which was aspirated with a Pasteur pipette and inoculated onto other monolayer cultures if further plaque passages were desired.

Temperature control. Incubators (Assmundson Aktiebolag-Assab Bacteriological Cabinet T-200) equipped with fans to insure the most uniform temperature throughout were kept at 23, 37, and 40°C for incubation

of plate cultures. Internal thermometers were used to check the temperature which varied by ±0.1°C. Culture tubes were kept in mergotherm water baths (H. Struers Chemiske Laboratories, Denmark), maintained at 23, 37 and 40°C and equipped with an agitating apparatus to maintain a uniform temperature (variations of ±0.02°C).

Monkey virulence tests. Cynomologous monkeys weighing 2.5 to 4 kg were inoculated with 0.5 to 1 ml of virus suspensions into one or other of the cerebral hemispheres by means of a No. 24 (1″) needle. The animals were then observed daily for evidence of paresis or paralysis and after 18-21 days were sacrificed. Histological examination of the formalin-preserved brain and spinal cord tissue began with sectioning of the thalamus to demonstrate the needle tract. If the tract was found, blocks were removed from the midbrain, medulla, cervical enlargement, and lumbar enlargement, and embedded in celloidin. After being sectioned and stained with thionine, every 10th section was examined microscopically for the presence of inflammatory lesions and neuronal destruction or other histological evidence of poliomyelitis infection.

Experimental. History of passages. Fig. 1 summarizes the schedule of tissue culture passages leading to development of the new type 3 strain (the WM-III virus). Material used to initiate the present passage series represented the currently used W-Fox vaccine strain (W-Fox vaccine pools in Fig. 1). It will be recalled that originally the W-Fox virus was isolated in tissue culture from an asymptomatic child in Louisiana by Dr. John P. Fox, and found to be of low pathogenicity for monkeys(10). In this laboratory (Fig. 1) the Fox strain was passed on monkey kidney monolayers for 6 serial single plaque passages: the sixth plaque passage was made into a monkey kidney pool for seed material, and as shown in Fig. 1 was used in the course of 4 further kidney tissue culture passages for preparation of live virus vaccines employed in vaccination programs of man in the United States, the Congo, Poland, Switzerland, and Croatia(1,2,7,12,13,14).



TCP = Serial passages in monkey kidney tissue culture tubes.

PP = Serial single plaque passages on monkey kidney tissue culture plates.

FIG. 1. Passage history of the WM-III strain.

Although W-Fox replicated relatively poorly in cultures kept at 40°C, results of comparative titration conducted at 37°C and 40°C indicated that the difference in reproductive capacity never exceeded the figure of 2.0-3.5 $\log_{10}$ TCD_{50} (Tables I and III). Usually after one human passage the virus changed to a strain which grew as well at 40°C as at 37°C (Table I).

On one occasion, however, growth of an excreted virus was impaired when the cultures were kept at 40°C and this virus strain was used to start a series of culture passages at 23°C following the technic originally suggested by Dubes and Wenner(15). During the first 2 passages no cytopathic effect was noted in tissue cultures and the material was passed blindly at 12-day intervals. On the 3rd tissue culture passage the virus produced a cytopathic effect 3-5 days after inoculation. Virus representing the 22nd tissue culture passage was plaqued on monolayers incubated at 23°C, and the clone grown for one passage in tissue culture tubes kept at 23°C (Fig. 1). Following that, 3 more plaque passages on monolayers were made at 23°C and the resulting strain passed for 10 tissue culture passages in tubes incubated this time at 37°C (Fig. 1), to provide the final material, which was named the WM-III strain.

The WM-III strain replicated equally well

TABLE III. Reproductive Capacity of WM-III in Culture Tubes Incubated at 23, 37 and 40°C.

Virus	$Log_{10}TCD_{50}$ at 23°C	37°C	40°C
W-Fox	<.0	7.7	4.7
H-24	<.0	8.2	8.0
WM-III	7.5	7.5	<.0

in cultures kept at 23 and 37°C but did not grow at 40°C (Table III). In contrast, growth of the virulent type 3 H-24 strain was unimpaired by incubation temperature of 40°C whereas the titer of the attenuated W-Fox was lower at 40°C than at 37°C; but the difference in titers, as mentioned before, did not exceed 3.0 $logs_{10}$. Both H-24 and W-Fox failed to grow at 23°C.

Infectivity of WM-III strain for man and stability after human intestinal passage. Nine infants, 4 premature and 5 full-term, at ages ranging from 3 days to 3 months were fed the WM-III strain by dropper in the amounts of $10^{4.2}$ to $10^{7.7}$ TCD_{50} (Table IV). Stools were collected twice weekly and processed as described previously(5). All infants excreted virus for one week or more. Fecal virus strains isolated 2 to 42 days after ingestion of the vaccine were titrated in cultures kept at 37 and 40°C, respectively, and on one occasion at 23°C. Virus in the original stool and after the first tissue culture passage at 37°C was used as inoculum. The results, summarized in Table IV, indicated that none of 9 feedings resulted in excretion of an rct/40°C+ virus, in contrast to the results obtained with W-Fox virus (Table I). The virus did not grow at all or grew rather poorly in cultures kept at 40°C. The rct/40°C– character was retained by the excreted WM-III strains regardless of the time interval elapsing between virus administration and its isolation from the feces. In one case

TABLE IV. Reproductive Capacity at 37 and 40°C of Fecal Virus Isolates from Infants Fed the WM-III Strains.

Subject No.	Dose fed log TCD_{50}	Virus isolated post-vacc. day	Material tested	$Log_{10}TCD_{50}$ 37°C	40°C	$Log_{10}TCD_{50}$ 37°C minus $Log_{10}TCD_{50}$ 40°C
1	7.7	6	St	4.4	<1.4	>3.0
			1TC*	8.2	5.2	3.0
		30	St	1.7	<1.5	> .2
			1TC	7.2	<2.5	>4.7
2	7.5	2	St	2.7	<1.5	>1.2
			1TC	7.7	<2.5	>5.2
		9	St	3.5	<1.5	>2.0
3	6.5	9	St	4.5	<1.5	>3.0
			1TC	7.5	3.7	3.8
		19	St	2.5	<1.5	>1.0
4	6.5	9	St	3.7	<1.5	>2.2
			1TC	7.5	<2.5	>5.0
		23	St	4.2	<1.5	>2.7
			1TC	7.2	<2.5	>4.7
5	5.5	10	St	2.5	<1.5	>1.0
6	5.5	4	St	2.2	<1.5	> .7
			1TC	6.7	<2.5	>4.2
7	5.5	18	St	2.2	<1.5	> .7
			1TC	8.2	4.2	4.0
		42	St	1.7	<1.5	> .2
			1TC	6.7	<2.5	>4.2
8	4.2	5	St	2.5	<1.5	>1.0
			1TC	7.2	<2.5	>4.7
9	4.2	5	St	5.2	<1.5	>3.7
			1TC	7.7	<2.5	>5.2

* Gave a titer of $10^{7.2}$ TCD_{50} at 23°C.

St = Stool. 1TC = First tissue culture passage in monkey kidney cells.

TABLE V. Monkey Neurovirulence (Intracerebral) of WM-III Strain and Its Human Passages in Comparison to W-Fox.

Virus	Dose $Log_{10}TCD_{50}$	Ratio of monkeys Paralyzed	With lesions
W-Fox	7.2	3/10	4/10
WM-III	6.5	0/5	0/5
A*	7.9	0/5	1?/5
B*	7.5	0/5	0/5

* First tissue culture passage of strains isolated from subjects fed WM-III.

in which a titration was performed at 23°C the fecal WM-III virus retained its ability to reproduce at that temperature. The 2 viruses isolated 9 days after vaccination from infants 3 and 4 were tested by a newly discovered marker, acid sensitivity(16). In this test viruses are incubated in presence of 0.35 N HCl for 3 hours at 23°C. W-Fox and WM-III are both highly susceptible to the action of acid, whereas the virulent H-24 maintains its titer in acid at the same level as at neutral pH. Excreted strains of W-Fox submitted to this test showed acid resistance in 4 cases, intermediate resistance in 2 cases, and susceptibility only in one instance. Two WM-III fecal strains which were tested, however, remained acid-susceptible.

Monkey neurovirulence test. Table V summarizes results of clinical and histologic observation of monkeys injected intracerebrally with the WM-III virus and with material representing passage of the strain through the human intestinal tract. No clinical signs of illness were noted in 5 monkeys injected with WM-III virus or in 10 monkeys inoculated with material obtained from 2 virus-fed subjects. Examination of over 150 sections of central nervous system from each monkey failed to reveal histologic changes in 14 of 15 monkeys. Only in the case of one monkey were scattered inflammatory lesions with an occasional loss of neurons observed. These results differ markedly from those obtained with the W-Fox strain, which are included in Table V for comparative purposes.

Discussion. This paper reports the development of a new type 3 attenuated poliovirus, named WM-III, which was derived from the W-Fox vaccine strain after 22 passages in tissue culture tubes, and 4 passages by plaquing and clone selection at 23°C. To insure its infectivity for the human gastrointestinal tract the final virus pool was made after 10 tube culture passages at 37°C. This material had no ability to grow at 40°C and was not neurovirulent for monkeys injected intracerebrally, in contrast to the original W-Fox strain. The results obtained with other sub-lines of W-Fox strain, as well as studies on the mechanism of cold adaptation will be reported separately(17).

The genetic stability of WM-III virus was demonstrated through its study after passage through the intestinal tract of a limited number of infants fed different quantities of the strain. The extracted virus showed poor capacity to replicate in tissue cultures incubated at 40°C (rct/40°C– character), which in 2 cases tested, retained their acid sensitivity. In addition, fecal viruses isolated from 2 vaccinated cases were found to be non-pathogenic for monkeys injected intracerebrally. Stability in relation to 3 markers makes the WM-III strain quite different from the 2 other attenuated type 3 strains investigated in the same way, as pointed out in Tables I and II(1-5). In addition to changes in *in vitro* markers, the excreted viruses derived from feeding of Leon KP-34 and W-Fox were found to be more virulent for monkeys injected intracerebrally(1-5). Melnick and Benyesh-Melnick(3), for example, reported paralysis and histologic lesions of poliomyelitis in 26 out of 52 monkeys inoculated with virus isolated from subjects fed the Leon KP-34 strain. The vaccine strain itself consistently produced asymptomatic infection.

More trials in man are required to evaluate fully the antigenicity of the WM-III strain, but the results obtained so far suggest that the high infectiousness of the W-Fox for human intestinal tract has been retained.

In view of the dissatisfaction with the stability of the available type 3 strains that has been expressed(3,8,18) and in view of a statement by the Committee on Live Poliovirus Vaccine of the Surgeon General of U.S. Public Health Service(19), and assuming

later studies confirm our results, the WM-III strain is the type 3 virus of choice for production of attenuated poliovirus vaccine.

Summary. Development of a new type 3 attenuated poliovirus (WM-III) is described. Starting with the W-Fox strain, passage and clone selection at 23°C were employed to develop a strain with improved stability after human intestinal passage. The end result was a virus which had no capacity to grow at 40°C and was not virulent for monkeys injected intracerebrally. The results of tests of fecal virus strains isolated from infants fed the WM-III strain did not show the instability in relation to laboratory markers that has been reported with other attenuated type 3 viruses.

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Studies of Inhibitory Effect of Ammonium Ions in Several Virus-Tissue Culture Systems. (26770)

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It was observed(1) that ammonium ions in trace amounts caused a very marked suppression of influenza virus replication in a dog kidney tissue culture system. This suppression was accompanied by a complete protection of the cells from the cytopathic effects of the virus. Similar results have been observed independently by Eaton and Scala (2) with both influenza and Newcastle disease virus in Krebs 2 ascites tumor cells. Since this unique inhibition may have a significant effect on many types of virus-host

cell studied it was considered important to determine the range of viruses and cell systems affected by this inhibitor.

This report presents results obtained with ammonium ions in test systems which include 10 virus species and 10 cell lines. In addition, ammonia was tested for inhibition against influenza virus in suspended allantoic membranes, embryonated eggs and in mice.

Materials and methods. The dog kidney cell culture has been described(1). Primary roller tube cultures of monkey, bovine, hamster and porcine kidney cells, and established cell lines, L-929, HeLa, Chang conjunctiva, human amnion (FL) and KB cells were obtained from a commercial laboratory.* Suspended allantoic membrane cultures were prepared by cutting the membrane from 11-day-old chick embryos into one cm square sections; the sections were washed several times in Hanks' balanced salt solution, pooled and distributed at random into T-30 flasks. Each flask contained 8 sections in 10 ml of BSS, and the membranes were maintained in suspension during incubation by a gentle action on a reciprocating shaker.

Viruses employed in this study were influenza, strains PR8, A′ and A_2, herpes simplex strain HF, Adenovirus type 4, Enders strain of mumps, Edmonston strain of measles, ECHO type 9, Parainfluenza type III, poliomyelitis type II (Lansing), vaccinia and Newcastle disease virus (NDV) Beaudette strain. All cultures were maintained in Eagle's minimum essential medium(3) from which glutamine was omitted and which was supplemented by 2.5% calf serum. Glutamine was omitted in order to eliminate, as much as possible, the presence of endogenous ammonia. Ammonia, in the form of NH_4Cl, was tested in each system by a tube dilution method. Two-fold dilutions of the compound in maintenance medium ranging from 1024 to 4.0 μg/ml were added to each culture and held one hour at 37°C. Virus was then added and the tubes were incubated at 37°C. The virus inoculum in each system was sufficient to give a 3-4+ cytopathic effect in 48 to 72 hours. Tubes were observed at the time of maximum CPE production in control tubes and, with influenza virus in allantoic membranes, hemagglutination (HA) titrations were conducted according to the method of Salk(4).

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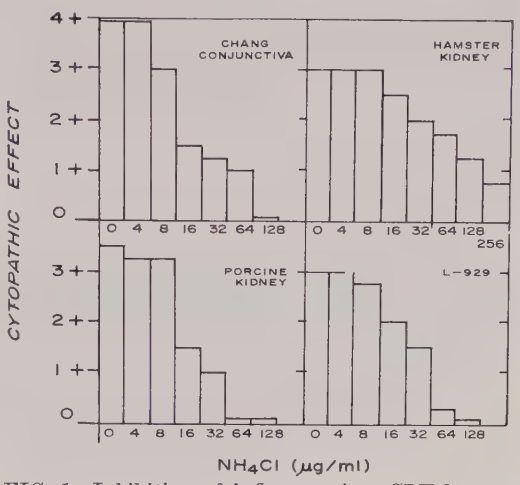

FIG. 1. Inhibition of influenza virus CPE by ammonia in four cell cultures.

Results. The inhibitory effect of ammonium ions on influenza virus in the dog kidney cell systems has been described(1). Inhibition of PR8 virus in 4 other cell systems is shown in Fig. 1. The inhibition of viral CPE was essetially the same in each cell line except hamster kidney cells which required higher concentrations of NH_4Cl for significant inhibition. The degree of protection afforded by ammonium ions may be seen in Fig. 2-4. These phase contrast photomicrographs of strain L-929 mouse fibroblasts clearly show the inhibition of influenza virus CPE by 40 μg/ml of NH_4Cl. Although a slight viral effect is apparent in the treated cells, in the form of vacuolization in the Golgi region, the cells are generally protected from the destructive effect of the virus.

The protective effect of ammonia was also shown in monkey kidney cells infected with influenza virus. Fig. 5 shows the effect of virus inoculum size on the inhibitory effect of NH_4Cl. The results indicate that a 100-fold change in inoculum size had little effect upon the amount of ammonia required for protection. From this experiment it can be concluded that inhibition is a result of the action of ammonia in the host cell and not upon the virus. This supports observations made in

the previous study where no direct inactivation of virus by NH_4Cl was demonstrated (1). Two other strains of influenza virus, A′ and A_2, were also studied in the monkey kidney cell system and essentially similar results were obtained.

To determine the effect of ammonia on influenza virus in more complex systems, tests were conducted in suspended chick allantoic membrane cultures, intact chick embryos and in mice. In the suspended membrane cultures virus production was measured by HA titrations and the effect of ammonia is shown in Fig. 6. Ammonium chloride appeared to

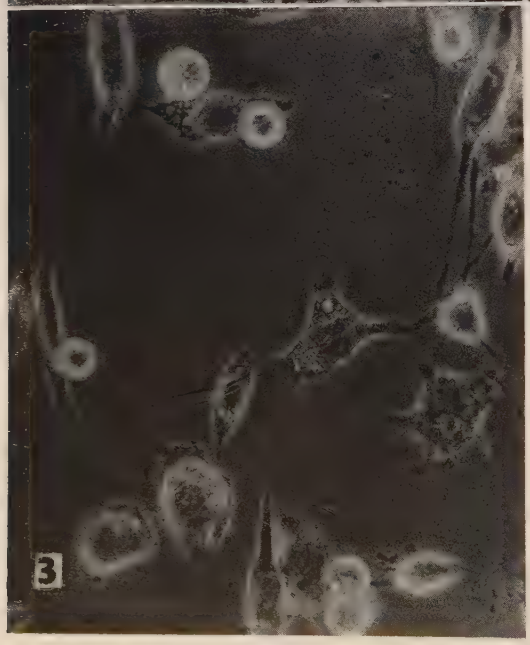

FIG. 2. Normal culture of L-929 cells. (Phase contrast, magnif. 320×.)

FIG. 3. Culture of L-929 cells similar to Fig. 1 48 hr after infection with 1000 $TCID_{50}$'s of influenza virus, PR8. Majority of cells have been destroyed as evidenced by the areas of cell debris. Remaining cells show severe viral CPE. (Phase contrast, magnif. 320×.)

FIG. 4. Culture of L-929 cells infected as in Fig. 2 but culture medium contained 40 μg/ml of NH_4Cl. Virus effect is reduced to moderate cytoplasmic vacuolization.

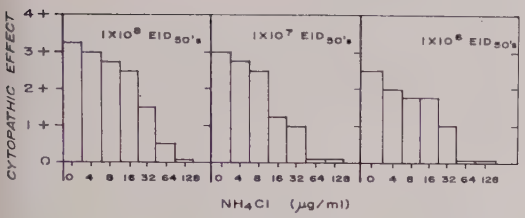

FIG. 5. Effect of virus inoculum size on inhibition by ammonia. Influenza virus PR8, in monkey kidney cell.

be toxic at levels between 500 and 1000 μg/ml, but significant virus inhibition was observed at levels as low as 16 μg/ml. Virus inhibition appears to be directly proportional to the amount of compound used.

In 10-day-old embryonated eggs a marginal and sporadic protective effect was observed when the eggs were treated with high concentrations of NH_4Cl. The compound is toxic at 40 mg/egg, but 4 to 8-fold reductions in HA titers at 24 hours were observed at levels as low as 5 mg/egg. An increase of 20 to 40% in survival rate was also observed. This phenomenon has been reported by Fauconnier(5) who attributes the effect to an acidification of the allantoic fluid. It is not known whether the inhibition observed in chick embryos is related to that observed in tissue culture.

Survival studies conducted in mice infected with approximately 10 LD_{50}'s of influenza virus, PR8, and treated with ammonium chloride administered orally, intraperitoneally or intranasally showed no protective effect.

Ammonium ions appeared to have no significant protective effects against the other viruses tested in this study. Only virus-cell line systems which produce an observable CPE were employed. In the case of herpes simplex virus indications of protection were observed in Chang conjunctiva, bovine and hamster kidney cells, but these effects were marginal and occurred only at near-toxic levels. Herpes simplex virus was not inhibited in dog kidney, HeLa or rabbit kidney cultures. Adenovirus type 4 was tested in dog kidney, HeLa, Chang conjunctiva and bovine kidney. Other systems studied include mumps in HeLa and Chang conjunctiva; measles in human amnion and KB; NDV in HeLa; ECHO-9, vaccinia and poliomyelitis in monkey kidney; and Parainfluenza type III in HeLa, Chang conjunctiva and bovine kidney cells.

Discussion. Although only a small number of viruses have been tested in this study, it appears that the inhibitory effect of ammonium ions is limited to influenza viruses. This is not in agreement with results reported by Eaton and Scala(2) on suppression of NDV in Krebs 2 cells. This discrepancy may be due to the difference in host cells or viruses used. We previously showed(1) that the effect cannot be explained in terms of direct virus inactivation or of interference with virus adsorption to the host cell. Thus, it was postulated that ammonia may interfere with some intracellular synthetic mechanism. The present study shows that cell protection is independent of virus inoculum size, which reinforces the concept of a compound-host cell reaction. If ammonia is interfering with some step in the synthesis of influenza virus, it may be concluded that this step is specific for influenza, and possibly NDV, since other viruses in the same cell line are not affected.

The marginal inhibition of influenza virus by NH_4Cl sometimes observed in the intact chick embryo and the definite suppression observed in suspended allantoic membrane are of interest. Although rather high concentrations are required in the intact embryonated egg, and Fauconnier(5) has attributed this

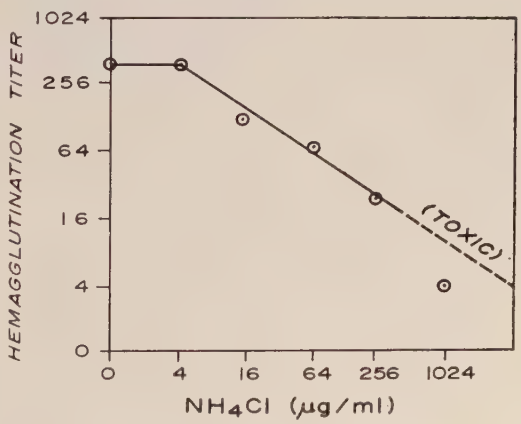

FIG. 6. Inhibition of influenza virus replication in suspended chorioallantoic membranes 48 hr after infection.

effect to a change in pH, it is conceivable that the tissue culture and the intact embryo results are related. Further studies in both systems may yield new information concerning the mechanism of action of the ammonia inhibition.

Summary. The antiviral effect of ammonium ions, previously shown in dog kidney cells against influenza virus, has been reexamined with 10 viruses in 10 tissue culture cell lines. Only influenza virus appeared to be affected by the inhibitor, which functioned in a number of cell systems. The effect was shown to be independent of virus inoculum size and therefore apparently on the host cell. The inhibition was demonstrated in suspended allantoic membrane cultures, and a marginal effect was also observed in intact chick embryos.

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Growth Characteristics of Ascitic Tumor Cells in the Heparinized Peritoneal Cavity of the Mouse.* (26771)

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Six years ago, at a conference on ascites tumors(1), we mentioned the occurrence of fluctuations in the amount of serous fluid and in its cellular content in mice bearing ascites tumor and given heparin intraperitoneally. Recently, we attempted to study the mechanism of this phenomenon by varying the method of administration, specifically by injecting heparin to some groups of animals at various intervals before tumor inoculation and to other groups after inoculation. In each experiment, the amount of ascitic fluid, its concentration of tumor cells, frequency of mitoses in these cells and extent of their implantation into the peritoneum were estimated, at requisite intervals (3 or 7 days) after inoculation. The results are reported and tentatively interpreted below.

Materials and methods. a) *Mouse and tumor strains.* Swiss Albino mice (Albino Farms, Red Bank, N. J.) of 24-27 g weight, mostly females, were used. As female mice with ascites tumors show a uniform and distinctive pattern of periuterine implant growth(1), they were preferred for our purposes. Krebs-2 ascites tumor was carried in our laboratory in serial intraperitoneal transfers. b) *Experimental technics.* Technics of tumor inoculation with requisite numbers of ascitic cells, of tumor cell counts, etc., have been described(2,3,4,5). Doses of approximately 10^5 tumor cells from ascitic fluid diluted (with .85% solution of NaCl) to a volume of .5 ml were injected intraperitoneally into lower *left* quadrant of the abdomen; the fluid subsequently accumulated in these mice was withdrawn by puncturing the *right* lower abdominal quadrant, thus avoiding trauma to granulating tissue and abundant bleeding (in heparinized animals) at sites of inoculation and of heparin injections (upper left quadrant). c) *Injections of heparin.* A standardized and commercially available solution of heparin (Panheparine Abbott) was used in our investigation. This solution, containing 1000 units (1 mg) per ml was diluted 1:20 with saline solution immediately before injections. Doses of .5 ml or 1.0 ml (25 to 50 units) were given before inoculation, but

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only .5 ml was given to inoculated animals which showed a tendency to bleed spontaneously during the first hours after injection. d) *Quantitative estimate of ascites tumor growth.* aa) *Amount of ascitic fluid.* The amount of exudate was estimated as proportionate to the volume of fluid obtained by insertion of a standard glass capillary in peritoneal wall: +++ = .5 ml or more; ++ = less than .5 but more than .1 ml; + = less than .1 ml; O = no fluid. The reliability of this estimate was shown by comparison with the amount of exudate measured at autopsy. bb) *Scale of peritoneal implantation*: no implant = 0; circular nodules only, 1 to 2 mm in diameter = +; 3 to 5 mm in larger diameter = ++; larger = +++. e) *Mitotic index counts.* Fluid smears were stained with Acetoorcein (2) for the purpose of counting mitoses. f) *Pattern of experiments.* aa) *Heparinization after tumor inoculation.* Three or 4 injections were given daily, starting on first day after inoculation. bb) *Heparinization before inoculation.* Three or 4 injections were given daily, the last 6 hours before inoculation. cc) *Combined method.* Heparin injections 48, 24, and 6 hours before inoculation and the 4th injection 24 hours after inoculation. dd) *Recording the results.* In one-half of the mice in each experimental and control group (inoculated but not heparinized), ascitic fluid was withdrawn for volume estimate and tumor cell counts on the 4th day after inoculation, and the autopsies for recording implantation were performed on the 5th day. In the other half of the animals the fluid was withdrawn and the mice were sacrificed on the 8th day.

Results. Mice were divided in 3 series according to 3 patterns of heparinization outlined in Materials and methods. Each series included 3 groups, each of 20 mice: 2 groups were inoculated on the same date and heparinized by the same plan, but examined for records at different time intervals, on the 4th or the 8th day. The third group, inoculated on the same date but not heparinized served as control. The data obtained in all groups of mice were tabulated in Table I and illustrated in Fig. 1-6.

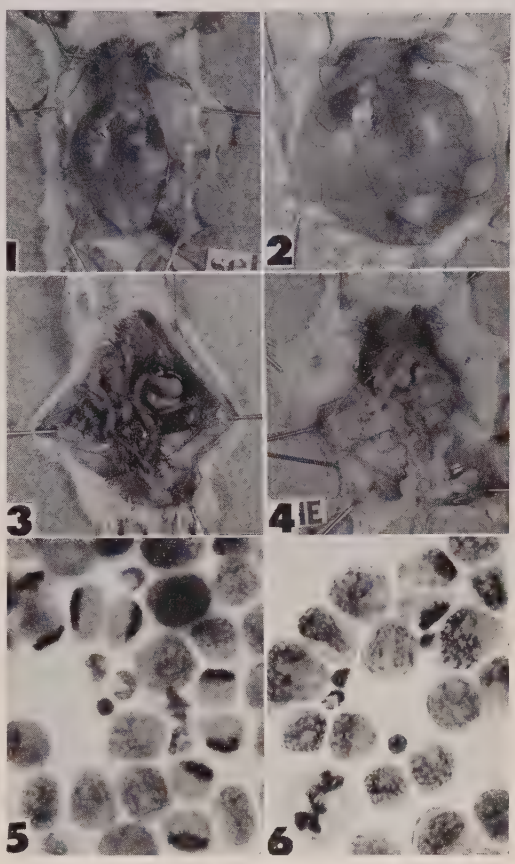

FIG. 1, 3, and 5. Mice given 50 units of heparin intraper., 48, 24, and 6 hr before inoculation of Krebs-2 ascites tumor.

FIG. 2, 4, and 6. Controls inoculated simultaneously with exp. mice but not heparinized.

FIG. 1-4. Mice examined on 8th day after inoculation.

FIG. 5-6. On 5th day.

Note difference in amount of fluid (Fig. 1-2) and in extent of implantation (Fig. 3-4); implant at site of inoculation in Fig. 1; frequency of mitoses in anaphase shape in Fig. 5 (×400, Aceto-orcein).

The data in the various series and groups suggested the following: 1. The combined method of heparinization of mice before and after ascites tumor cell inoculation (Series III) inhibited consistently and significantly all 3 phenomena of growing ascites tumor, *i.e.,* fluid accumulation (Fig. 1 as compared with Fig. 2), increase in concentration of free tumor cells and their implantation in the peritoneum (Fig. 3 as compared with Fig. 4). Heparinization only before inoculation (Series II) produced similar effects but mostly at a lower scale, and the same treatment applied

TABLE I. Free Growth and Implantation of Ascitic Tumor Cells in Heparinized Swiss Albino Mice Inoculated with Krebs-2 Ascites Tumor (20 Mice in Each Group).

Series	Group	Timing of heparinization*	Interval between inoc. and recording of results (days)	Results (avg and variation extremes in each group)			
				Amt of ascitic fluid†	Conc. of tumor cells in fluid (thousands/mm³)	Mitotic index of tumor cells	Extent of implant growth†
I	1	Heparinization starting 24 hr after inoc.	3	++ +-+++	42.3 36.1-51.4	7.7 5.2-6.8	+ +-++
	2	Controls (non-heparinized)	"	++ ++-+++	48.2 40.8-56.4	7.1 5.6-9.1	++ +-+++
	3	As Group 1	7	++ +-+++	48.5 42.2-54.8	7.8 4.9-9.4	+ +-++
	4	Controls	"	+++ ++-+++	52.7 43.2-59.8	6.5 4.4-8.8	++ ++-++
II	1	Heparinization ending 6 hr before inoc.	3	+ +-++	46.9 39.2-55.5 56% in anaphase	10.2 7.8-12.5	+ 0-+
	2	Controls	"	++ ++-+++	57.2 41.4-67.5 28% in anaphase	7.1 4.9-9.4	++ ++-+++
	3	As Group 1	7	+ +-++	49.4 43.2-54.2	6.9 5.6-9.2	+ 0-+
	4	Controls	"	++ ++-+++	56.6 44.9-65.1	7.4 5.1-8.8	+++ ++-+++
III	1	As Series II and one inj. 24 hr after inoc.	3	+ 0-++	47.7 37.2-52.3 48% in anaphase	12.1 8.9-15.2	0 0-+
	2	Controls	"	++ +-+++	59.7 40.2-67.4 2% in anaphase	7.2 6.2-9.4	++ +-++
	3	As Group 1	7	+ +-++	41.2 29.5-54.2	7.4 6.2-8.8	+ 0-+
	4	Controls	"	++ ++-+++	59.1 49.1-69.5	7.2 5.5-9.1	+++ ++-+++

* 3 or 4 daily inj. of 25 or 50 units of heparin.
† For estimate of fluid volume and scale of implants, see *Materials and methods.*

entirely after inoculation (Series I) resulted only in irregular fluctuations in the level of these phenomena.

2. Mitotic index of tumor cells in ascitic fluid was higher in heparinized mice of Series II and III on the 4th day after inoculation than in controls, but was lower on the 8th day, suggesting that the majority of mitotic cells had not completed their division; the high proportion of anaphase figures suggested arrest of the mitotic cycle in this stage (Fig. 5 as compared with Fig. 6).

3. Concentration of ascitic tumor cells was considerably decreased in Series II and III, in spite of a substantial decrease in the volume of their diluent, *i.e.*, ascitic fluid as compared with controls. Thus, there was greater reduction in number of ascitic cells than in volume of fluid, *i.e.*, the process of cell proliferation was inhibited more strongly than the process of fluid exudation.

4. Some effects of heparin as recorded on the 4th day were apparently reversed by the 8th day; amount of fluid increased through slow accumulation of scanty but continuously exudated fluid, which may be in part responsible, for the further drop in tumor cell concentration. Some implants appeared, mainly at the site of abdominal puncture (Fig. 3), suggesting that some inoculated tumor cells invading the puncture wound in this location escaped the effect of heparinization. However, the surface of serosa remained free from implants. Thus, it may be emphasized that of all principal features of growing ascites tumors, only implantation

was inhibited completely in several heparinized animals (Series II and III), and for an indefinite period.

Discussion. There are varying opinions as to the nature and mechanism of ascitic fluid exudation from vessels of the peritoneum which are invaded by inoculated tumor cells (1,6,7). However, it is a consensus that this invasion is a prerequisite for appearance of intracavitary fluid and a starting point for free growth of tumor cells. In our experiment, heparinization of the peritoneal cavity inhibited implantation of cells into serosa more consistently and more completely than it inhibited other features of ascites tumor growth. Accordingly, prevention of tumor cell implantation may be considered as the primary effect of heparinization, which was responsible for other effects of this procedure.

Implantation of tumor cells invading the peritoneal serosa occurs through their penetration into connective tissue of subserosa made available by trauma to the epithelial surface(8). It has been repeatedly reported that both in human transcoelomic metastases (9) and in analogous phenomena labeled as ascites tumors in rats and mice(10,11,12) the first step in the process of implantation is the embedding of tumor cells by fibrin clotted at the site of slight injury of peritoneal serosa by tumor cells. "Next to the cancer cells themselves, fibrin is the most important factor in the life history of peritoneal implantation. It is fibrin which holds the cancer cells in place by splinting them against the denuded peritoneum. Fibrin is both the temporary framework and the scaffolding of the organized implants"(9). Consideration of these phenomena may provide interpretation of our findings reported above.

It is known(12) that heparin is not only anti-prothrombin but also anti-thrombin, preventing thrombin from converting fibrinogen to fibrin. Its effect *in vivo* is more lasting than *in vitro* and our daily injections induced a continuous heparinization (as was shown by the prolonged fluid state of the shed blood). Clinical experience has shown that continuous heparinization may prevent accretion of fresh fibrin clot and thus allow time for its resorption. It may be presumed that in our experiments a similar mechanism released tumor cells embedded in the fibrin clot attached to peritoneum, and that damaged serosa healed after elimination of fibrin clot. If this is correct, the currently applied postoperative heparinization for prevention of thromboses may be of additional help in cancerous patients by preventing implantation of locally spilled or systemically circulating tumor cells.

It should also be noted that all of the mast cells which are an abundant source of heparin disappear from the peritoneal fluid of mice within 2 or 3 days after inoculation of tumor cells(2) and reappear only in cases of complete regression (by chemical or radioactive agents) of tumor growth. This phenomenon, suggesting antagonism between mast cell migration from connective tissue to peritoneal cavity and invasion of serosa by tumor cells, is being investigated in the light of our experiments.

Summary and conclusions. 1. In a large proportion of Swiss Albino mice which had received 3 daily intraperitoneal injections of heparin (25 or 50 units) immediately before inoculation of Krebs-2 ascites tumor, the amount of ascitic fluid and its concentration of tumor cells were consistently and significantly lower than in controls. 2. Macroscopically visible implantation of ascitic cells from the fluid into the peritoneum was considerably reduced and sometimes completely absent in overwhelming majority of heparinized mice (Series II). 3. An additional injection of heparin (twenty-four hours after inoculation) increased these effects (Series III) while heparinization given entirely after inoculation (Series I) induced only wide fluctuations in the extent of ascites tumor growth. 4. Accordingly, inhibition of implantation was considered to be the primary and direct effect of heparinization, which may account for reduction of the amount of ascitic fluid and of its concentration of tumor cells as secondary effects. 5. It was accepted, in the light of research by other investigators and by ourselves, that embedding of free tumor cells within a fibrin clot from the cavitary fluid initiated their implantation into

the serosa and that preventive heparinization arrested implantation in this early stage.

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A Method for Determination of Parathyroid Secretion Rate in the Rat.* (26772)

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While secretion rates of some endocrine glands, notably thyroid, have been measured by a variety of technics, virtually no attempt has been made to estimate parathyroid hormone secretion rate (PSR) in individual small laboratory animals. This is partly due to the difficulty in obtaining adequate serial samples of blood in the same animal and, in part, to a failure to develop technics comparable to those utilized in determining the thyroxine secretion rate. The availability of an ultramicro method for determination of serum calcium(1) has led to an attempt to determine PSR in individual rats, based on a principle similar to that used for measuring thyroxine secretion rate(2). This report presents the feasibility of such a technic.

Experimental. One hundred and thirty-three female rats of a local strain, each weighing about 200 g, were housed in cages maintained at uniform temperature (78 ± 2°F) and illuminated only during daylight hours. They were fed Purina Laboratory chow *ad libitum* and water was available at all times. The rats were thyroparathyroidectomized (TPEx) surgically, under ether anesthesia. Completeness of TPEx was checked by administering I^{131}, and when radioactivity over the neck region was less than background this indicated an absence of thyroid I^{131} pick-up. One day prior to TPEx the laboratory chow was replaced by a diet in which calcium content was less than .016%, in order to prevent calcium in the feed from affecting serum calcium levels following TPEx. Serum calcium was determined by a chelating procedure utilizing ethyelene diamine tetra-acetate (EDTA) as the complexing agent. As little as 20 lambda (20 μl) of serum was found to be sufficient for each calcium determination. Blood samples were collected from the tail vein directly into polyethyelene microcentrifuge tubes and centrifuged in a Beckman microfuge. Twenty lambda of serum were titrated against EDTA in a microtitrator in a highly alkaline medium utilizing calcein as a indicator. The endpoint of titration was represented by the disappearance of the blue-green color. With little practice it was possible to distinguish this endpoint clearly. An ultraviolet light source may also be used to aid in distinguishing the endpoint sharply. Serum calcium determinations by this technic checked against classical flame photometry methods were found to be well within limits of expected experimental error. Re-

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peated serum calcium determinations (at least 6-8) with 20 lambda of serum from the same rat yielded a variation of only ± 0.4 mg %.

Experimental procedure consisted in determining serum calcium levels prior to TPEx followed by serial determinations thereafter at 3, 6, 9, 12 and 24 hours after operation. After TPEx, when serum calcium had reached its lowest level, parathyroid hormone (PTH)† was injected subcutaneously into each rat in graded amounts of 25 units. PTH administration was discontinued when the post TPEx serum calcium reached the pre TPEx level. At this point it was assumed that sufficient PTH had been administered to maintain the serum calcium at a normal level. However, in many individuals serum calcium again declined within 24 hours after final PTH injection. Thus, the duration of PTH replacement was also determined in each animal.

Results. Serum calcium in individual rats before TPEx varied between 9 and 12 mg %. After TPEx serum calcium generally declined to its lowest level in about 6 hours, although periods up to 18 hours were noted in some rats. The percentage decline in serum calcium following TPEx varied from 0 to 51% of the initial value. In 10 rats as little as 5 I.U. of PTH were sufficient to elevate post TPEx serum calcium to the pre TPEx level while 25 I.U. were required in 20 other animals. In the majority of rats (80), 45 I.U. were necessary, while in another 10 animals the requirement was as high as 65 I.U. When the frequency distribution of the PTH requirement was plotted (Fig. 1), a 13-fold variation in individual rats was found. The final injection of PTH replacement (*i.e.*, the amount necessary to equate pre and post TPEx levels) lasted for an average of 10 hours, with a range from 8 to 12 hours. This was shown by the precipitous decline of serum calcium as the effect of PTH was lost. In certain rats in which excess PTH had been administered, serum calcium soared to levels far in excess of the pre TPEx value (Fig. 2). In such cases the average of the terminal and the preceding PTH injections was taken as the level of adequate replacement.

Discussion. As far as we are aware, this represents the first attempt to determine the quantity of PTH necessary to maintain normal serum calcium levels following TPEx. Individual estimations are of greater signifi-

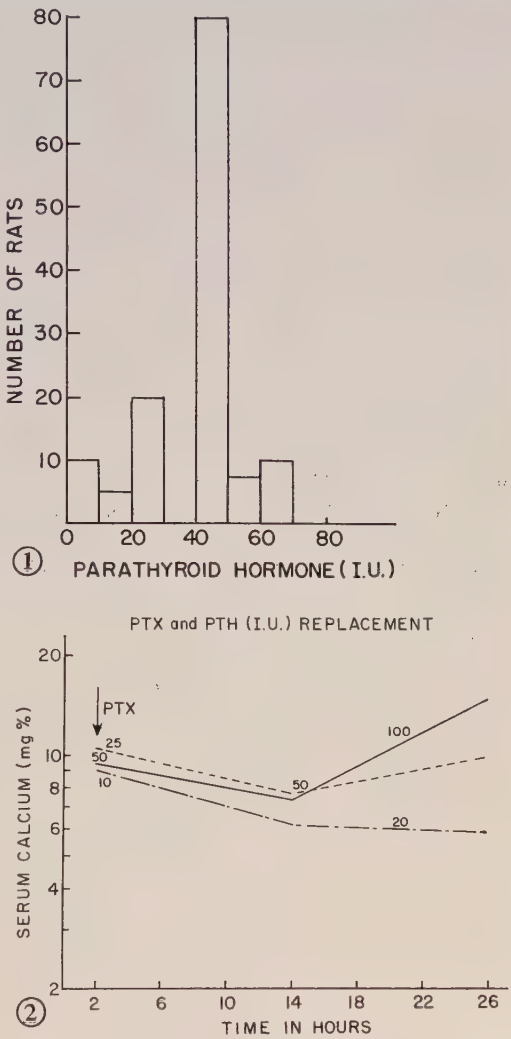

FIG. 1. Frequency distribution of PTH replacement in I.U./100 g body wt in individual rats following TPEx. While there was a 13-fold individual variation, mean PTH replacement was 40 I.U.

FIG. 2. Illustration of the principle of PTH replacement in individual rats. PTH is administered in increasing amounts following TPEx until post and pre TPEx serum calcium levels become equal. Administration of excess or insufficient amounts of PTH will not equate post and pre TPEx serum calcium levels.

† Obtained from Eli Lilly Laboratories, Indianapolis, Ind.

cance than the mean in interpretation of physiological variations as well as the influence of interrelated hormones and associated phenomena under controlled conditions of temperature and nutrition. This is particularly evident since a 13-fold variation was found in the individual PTH requirement following TPEx. Such variability can be compared with a 6-fold variation in the thyroxine secretion rate of rats(3) and with an 11-fold variation in the same determination in cattle(4). In certain rats the decline in serum calcium following TPEx was almost negligible.

Four possible reasons for such variations are: (a) Presence of accessory parathyroid tissue; (b) individual differences in parathyroid hormone secretion rate; (c) absorption of calcium from the gut, and (d) interaction of parathyroid hormone with other hormones to nullify its effect. Since the completeness of TPEx was checked in each individual, and since accessory parathyroid tissue is seldom present in rats(5), the first reason appears unlikely. The third possibility is not likely, since the commercial chow was replaced by a low calcium diet prior to TPEx and continued to the end of experiment. As regards the interaction of other hormones, the fact that these animals are athyroid is of some significance, since it has been observed that thyroxine may influence reabsorption of phosphate in the kidney and thus indirectly affect serum calcium level(6). A secondary influence of other hormones on serum calcium levels, notably those of the adrenal cortex, has not been eliminated in these studies. Since PTH was administered in increments of 25 I.U. the possibility also exists that slightly smaller increments of PTH replacement might give more accurate results. Increments of 25 I.U. were chosen to minimize the number of blood samples required to determine replacement levels. Furthermore, different levels of replacement might be obtained if routes other than the subcutaneous route were employed. Different values might also be obtained with hormone of greater purity. Despite these limitations, the present experiments indicate the validity of the method, although further studies are necessary to establish more absolute values.

Since it was found that a single adequate injection of PTH in TPEx rats maintained the serum calcium level for an average of only 10 hours, this hormone would appear to have only a short half-life. While the level of PTH replacement as determined in this study is in the general range reported in rats by Munson(7) and by Berswordt and Turner (8), if one considers the average duration of effect of parathyroid hormone (10 hours), it would appear necessary to multiply the estimated values in the present study by a factor of 2.4 to arrive at a 24-hour parathyroid hormone output. The range of PTH secreted over a 24-hour period would then be 12-156 I.U. per day with a mean of 96 I.U. Since these rats received a low calcium diet for only one day prior to TPEx, it can be assumed that the parathyroids were in an essentially normal state at time of removal and that the data represent normal individual physiological variations.

Summary. A technic is described in rats for individual determination of the 24-hour parathyroid hormone secretion rate (PSR). This method utilizes the subcutaneous administration of increasing amounts of parathyroid hormone (PTH) following thyroparathyroidectomy (TPEx) until pre and post TPEx levels of serum calcium are approximately equal. There were individual variations in the time required for serum calcium to reach its lowest level following TPEx and also in the quantity of PTH required to elevate serum calcium to the pre TPEx level. These probably represent individual variations in PSR. The average duration of effect of a single adequate PTH replacement was 10 hours, and from this the average PTH secretion over a 24-hour period was calculated to be 96 I.U.

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Plasma Deoxyribonuclease and the Quantitative Effects of Injected DNA.* (26773)

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It has been reported from this laboratory that a single injection of purified, undenatured deoxyribonucleic acid (DNA) produced in rabbits a significant decrease in serum cholesterol lasting at least 2 years(1,2,3). DNA preparations from homologous and heterologous mammalian tissues were both effective. The lowered mean serum cholesterol largely reflected the decline in cholesterol levels of those animals with high normal values. Quantitative studies of varying the amount of DNA injected over a 50-fold range showed a significant dose-response relationship(3).

One problem which arises in interpreting the quantitative data is that these animals have a circulating, potent plasma deoxyribonuclease. Some of the injected DNA molecules must be inactivated, denatured, or even degraded prior to their absorption by the cells of the organism. The enzyme circulating in the blood is similar to the pancreatic deoxyribonuclease I in its properties and requirements for activity(4). Mg^{++} ion is an essential activator and citrate is an effective inhibitor which acts by binding the Mg^{++}(5). The present study describes the inhibition *in vivo* of the plasma deoxyribonuclease by sodium citrate and the effect of this inhibition on the physiologic activity of injected DNA.

Methods. DNA used for injection was prepared from fresh beef liver by the mild procedures previously described(1), *i.e.*, extraction with high ionic strength salt and deproteinization with chloroform. The N/P ratio was 1.75. The fibres were dissolved in either isotonic saline or .015M sodium citrate plus isotonic saline, and injected immediately. The DNA used as the substrate for the enzyme determinations was prepared in identical fashion and dissolved in isotonic saline to a concentration of 0.15%. This was heated to 60°C for 30 minutes to destroy any residual deoxyribonuclease, placed in 10 ml aliquots and stored frozen at –15°C. The relative viscosity was 4.2.

The bloods were drawn into heparin in an ice bath, then centrifuged at 3000 r.p.m. for 30′ in a refrigerated centrifuge at 2°C. The supernatant plasma, apparently free of formed elements, was removed and stored frozen at –15°C. Deoxyribonuclease activity was determined by adding 1 ml of the heparinized rabbit plasma and 0.1 ml .02M $MgCl_2$ to 2 ml of the DNA substrate solution in an Ostwald viscosimeter at 37°C. The run-through time of the viscosimeters was approximately 20 seconds. Following a mixing period of one minute, viscosity measurements were made at 3 minute intervals for 30 minutes. Under these conditions, the straight-line portion of the curve lasted 18 minutes.

Total serum cholesterol was determined by a Bloor(6) procedure with 10 ml of the Bloor's reagent containing the extracted serum being taken to dryness.

Albino rabbits of the New Zealand strain weighing 2-3 kg were kept in temperature-controlled, air-conditioned, separate quarters and fed a standard chow *ad libitum*. All the

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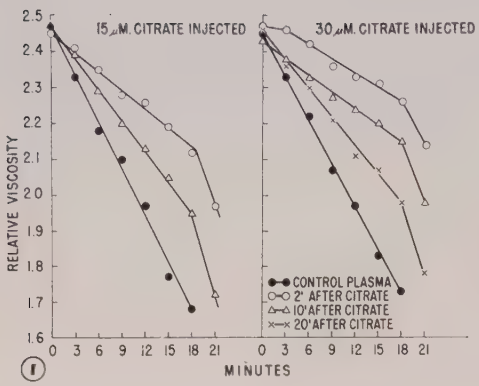
† Established Investigator, American Heart Assn.



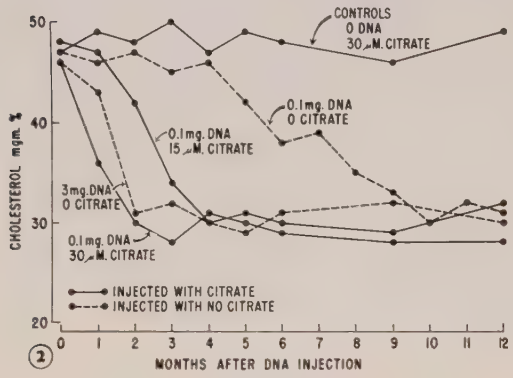


FIG. 1. Altered plasma deoxyribonuclease activity following sodium citrate inj.

FIG. 2. Avg serum cholesterol levels following inj. of DNA, with and without sodium citrate.

animals were kept at least 6 weeks in these quarters, prior to use, to permit them to adapt to the new, constant environment. Only those rabbits were used which ate well, showed a steady gain in weight and no evidence of disease.

Results. Sodium citrate solution (.015M $Na_3C_6H_5O_7$ + .15M NaCl) was rapidly injected intravenously into rabbits in amounts ranging from 9 μMoles to 150 μMoles. The animals were bled from the ear vein into heparin prior to injection and at 2′, 10′, 20′ and 30′ intervals after the citrate injection. The plasma samples were tested for their deoxyribonuclease activity as indicated above. A transient, but significant, inhibition of plasma deoxyribonuclease was observed in all the animals injected with the citrate solution. Fig. 1 demonstrates typical changes in 2 rabbits; one injected with 15 μMoles and one with 30 μMoles of citrate. In the animal receiving 15 μMoles there is a deoxyribonuclease inhibition of about 40%, 2′ after citrate injection and a gradual return to control levels within 20′. The plasma of the rabbit injected with 30 μMoles showed 60% inhibition and 30′ were required to return to the control activity. Injection of greater amounts of citrate resulted in the same magnitude of enzyme inhibition (60%) with the effect lasting longer.

The data indicated that *in vivo* plasma deoxyribonuclease activity was inhibited by injection of sodium citrate which, presumably, removed some of the necessary Mg^{++} ions. The question arose as to whether these small quantities of citrate, when diluted hundredfold into the total plasma volume, were capable of binding sufficient Mg^{++} to produce the observed inhibition. To check this, studies were done in which an equal volume of .015M $MgCl_2$ was injected separately into another vein immediately after the .015M sodium citrate injection. The injection of the small quantity of Mg^{++} completely prevented the citrate-induced enzyme inhibition.

The effect of plasma deoxyribonuclease inhibition on the quantitative physiologic activity of injected DNA was then determined. The same preparation of heterologous DNA was injected intravenously into several groups of rabbits, with and without sodium citrate, and the effects on serum cholesterol levels were studied. The animals were divided into 5 experimental groups with 10 normal, healthy rabbits in each group receiving the following injection: 1) controls—30 μMoles of sodium citrate in isotonic saline, 2) 0.1 mg DNA in saline, 3) 3 mg DNA in saline, 4) 0.1 mg DNA plus 15 μMoles sodium citrate in saline, 5) 0.1 mg DNA plus 30 μMoles sodium citrate in saline. The DNA injections did not produce any toxic effects, acute or chronic. There was no difference in nutrition, weight gain or general health between injected and control groups. The animals were bled from the ear vein prior to injection and at monthly intervals thereafter for 12 months. Total serum cholesterol was measured on all the bloods.

Fig. 2 demonstrates the changes in the average serum cholesterol level of the 5

groups of rabbits over a period of one year after the injections. The altered serum cholesterol appeared more rapidly in those animals receiving DNA and citrate. The changes observed in the group injected with 0.1 mg DNA and 30 μMoles citrate were comparable to those of the group injected with 30 times as much DNA without citrate. Previous quantitative studies(3) had shown that over this same dosage range the time required for the appearance of the serum cholesterol changes varied inversely with the amount of DNA injected. Therefore, it appeared that *in vivo* inhibition of the plasma deoxyribonuclease by sodium citrate in this experiment had increased 30-fold the effectiveness of the injected DNA.

Discussion. The data presented indicate that injection of sodium citrate produces a significant *in vivo* inhibition of the deoxyribonuclease circulating in blood. The effectiveness of the injected DNA in lowering serum cholesterol levels was markedly increased when it was injected together with citrate. Injection of 0.1 mg DNA with a measurable, transient, enzyme inhibition lasting 30 minutes resulted in quantitative effects comparable to 3 mg DNA injected without citrate. This 30-fold greater effect suggests that the DNA injected without citrate in this, and in previous studies(1,3), has been largely inactivated (at least 97%) in the bloodstream prior to absorption into cells. It also serves to emphasize the exceedingly small quantities of "active" DNA needed to induce the prolonged alterations of serum cholesterol regulation.

It is noteworthy that a 2-fold inhibition of the plasma deoxyribonuclease as determined by viscosity measurements was associated with a 30-fold increase in physiologic activity of the DNA. A similar disproportion between viscosity changes and biologic activity was observed by Zamenhof and his colleagues (7) in the effect of crystalline deoxyribonuclease on bacterial transforming DNA. Incubation of small amounts of the enzyme with the DNA resulted in loss of 100% of the biologic activity and only a 10% decrease in the viscosity.

Summary. Sodium citrate injected into rabbits in amounts ranging from 9 μMoles to 150 μMoles produces a transient, significant *in vivo* inhibition of the plasma deoxyribonuclease. Injection of heterologous DNA into rabbits induces a prolonged decrease in total serum cholesterol. 30 μMoles of citrate and 0.1 mg DNA, injected together, are quantitatively as effective as 3 mg DNA in altering the serum cholesterol regulation. The citrate-induced deoxyribonuclease inhibition increased the physiologic activity of the injected DNA as much as 30-fold.

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In vivo P^{32} Radioautography in Detecting Breast Cancer.* (26774)

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A method of *in vivo* radioautography of the stomach based on selective uptake of P^{32} by malignant tissue, and employing a balloon coated with a photosensitive emulsion, has been described(1,2). Adaptations of this method for various other organs are being explored. The purpose of this communication is to report upon our experience with

* This work was supported by a grant from Am. Cancer Soc. and Atomic Energy Commission.

the method in the recognition of breast cancer.

Method. Patients for this study have come from the in-patient services of the University of Minnesota Hospitals. They include a group of women and one man. In most instances, these patients had been admitted for investigation of breast masses. Usually, a normal contralateral breast has been studied as a control. Occasionally, our examination has concerned a patient in whom the diagnosis already was known on the basis of prior excisional biopsy. In such patients, frequently no tumor is detectable in the breast upon its removal, even on histologic examination. Two such patients are included in this study. In a few patients under scrutiny for evidences of a gastric lesion, breasts were screened simultaneously while P^{32} studies were underway.

The patient is admitted to the hospital in the afternoon and receives a large tracer dose of sodium radiophosphate (500 μc) intravenously. A shield composed of a photosensitive emulsion coated on a vinyl plastic backing is then immediately applied to the breast while the patient is in the dark room. A heavy dressing of compresses and elastic Ace bandages is then applied so that all possible sources for light leaks are blocked out. Flattening of the breasts to distribute tissue mass is accomplished by this dressing. The bandage is left in place overnight for 18 hours and removed in the morning. The appearance of a dark area on the breast mold after developing the emulsion by immersion in developer and fixing solutions suggests the presence of a malignancy, owing to the greater affinity of neoplastic tissue for P^{32} over the adjacent normal tissue.

It has been necessary to secure adequate separation of emulsion from the skin to preclude destruction of the former by perspiration or its adherence to the skin. For this reason, a thin layer of Saran-wrap is applied directly to the skin and the shield is applied over it. Lubrication of the Saran-wrap itself, with powder or with glycerine, insures that the emulsion will separate readily when the test is concluded.

Modifications in technic have all been related to determining a suitable substance for a shield backing. Rigid molds of vinyl plastic coated with emulsion and molds of softer plastic with emulsion coatings have been employed as well as coated sheets of latex rubber, and emulsion-coated balloons. Currently, balloons and molds of soft flexible plastic are under investigation. The emulsion can be kept within a balloon and thus be protected from the skin. A certain degree of conformity to the breast can be achieved by pressure on the balloon alone. The use of a flexible soft plastic backing is much more comfortable for the patient and easy to handle for the operator. This can be molded to the breast readily. The problem of protection of the emulsion from the skin with this preparation does still exist, however.

TABLE I.

No. of patients	Diagnosis	Operation	Autograph
4	Cancer	Rad. mast.	+
8	Benign disease	Biopsy	7 – 1 +*
5	" "	0	—
10	Normal	0	—
2	Cancer totally excised by prior biopsy	Rad. mast.	1 – 1 +†
2	Old mastectomy scar no recurrence	0	—

* 1 false positive-light leak.
† 1 false positive-fresh biopsy site.

All of the above preparations have been coated by the Eastman Kodak Co., Rochester, N. Y., who have also supplied the plastic backings. Latex sheets and balloons have been supplied by National Hygienic Products Co., Akron, Ohio.

Results. To date, 31 breasts have been studied successfully (Table I). Four cancers not previously operated upon all gave a positive response. Ten normal breasts gave negative results. Seven of eight breasts with benign disease confirmed by surgical removal and histologic examination likewise were negative. Five breasts believed on clinical grounds to be benign were negative on radioautographic study and have yet not been operated upon.

In 2 additional instances, old mastectomy wounds, without any physical evidence of re-

currence, were studied. Both of these studies were negative. In another instance, a false positive response was associated with a benign lesion and is felt to represent an artifactual light leak. In one other instance a positive study was obtained in a breast with a fresh biopsy wound. No residual tumor histologically was present in the breast. In the final instance, a negative study was obtained in a patient who had had an excisional biopsy some time before the study. No residual tumor was present in this breast either.

Discussion. P^{32} has been employed by Nakayama in detection of esophageal and gastric neoplasms by using a Geiger-Mueller tube for detection(3). Neoplasms of cervix and vagina have likewise been studied by Geiger-Mueller tube counting(4,5). There have been numerous studies on the use of this method to diagnose cancers of the breast (6-9). Accuracy has been reported as high as 85% to 90% in several studies, but the method is tedious and permits identification of only fairly large and superficial lesions. Radioautographic methods seem to be more successful because they allow a long exposure of the recording medium to the surface being studied. This permits identification of areas with only slightly increased radioisotope content. Statistical errors associated with short-term counting technics are also avoided. Therefore, we felt that a recording exposure of some length would allow us to detect lesions in the breast with more accuracy than that obtained with a counter. In addition, the entire breast is scanned for areas of increased P^{32} content.

This test, it must be emphasized, is as yet developmental. We are presenting our initial experiences with it, which are encouraging. The penetration of P^{32} beta particles in tissue is no longer than 0.7 cm; however, the breast, compressed with a dressing to a minimal thickness and studied over a period of 18 hours apparently concentrates enough beta particle activity and secondary radiation to permit small increases of P^{32} activity to be detected by a sensitive emulsion.

This technic will be expanded and, together with technics for screening of the stomach, cervix and colon which are being developed at this hospital may afford an opportunity for screening of many areas of the body with one dose of P^{32}.

Summary. A method of *in vivo* radioautography of the breast as a screening procedure for breast neoplasms, using P^{32} has been presented and discussed. Thirty-one cases with 2 false positive results and no false negative results have been presented.

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Immunization to Homologous Milk in the Rabbit.*† (26775)

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The production of experimental "auto-allergic" lesions involving many tissues(1-9) has led to the anticipation that "auto-allergies" affecting other tissues, which contain parenchymal elements or secretions separated from the blood stream, will be discovered. The breast is such a tissue, since it is an exocrine gland whose main products are secreted externally and are not found in the circulation under normal conditions(10). That animals can make an immune response to homologous breast secretions was shown by Lewis(11) who demonstrated antibody to homologous casein in goats after multiple intravenous injections of goat casein. The present paper describes an attempt to produce anti-milk antibodies and "auto-allergic" breast disease in rabbits by immunization with homologous milk or casein or autologous milk in Freund's adjuvant(12).

Materials and methods. Rabbit milk was pooled, defatted by centrifugation, and frozen until use. Casein was precipitated by adjusting the pH of a 1:4 milk dilution in saline to 4.5 with 0.5 N HCl(13). After centrifugation the supernate was decanted and used as the whey fraction. The precipitated casein was washed 2 times in ether and solubilized by the dropwise addition of 0.05 N NaOH. The pH was not allowed to go higher than 9.8 and was finally adjusted to 7.4. The solutions of casein had a purity of 86% by paper electrophoresis(14). Protein nitrogen was determined by a modification of the micro Kjeldahl technic(15). "Milk-adjuvant" was prepared by addition of 10 parts defatted milk (50% in saline) to 1 part Arlacel C and 9 parts Bayol F containing 2.0 mg killed tubercle bacilli per ml of final volume and homogenizing to a high viscosity in a Waring Blendor. "Casein-adjuvant" was prepared in the same way with casein solution containing 8.9 mg casein N/ml. The rabbits received weekly injections of the given adjuvant for 2 to 10 months, first in the rear foot pads (1 ml), next in the front foot pads (1 ml), and subsequently intradermally in the back (½ ml). All injection sites were remote from the breast area. Animals were bled prior to the first injection and at monthly intervals just prior to an injection. Two male rabbits were immunized with milk-adjuvant and tested for antibody to homologous milk, casein and whey by the precipitin technic(16), double diffusion in agar(17), and hemagglutination. The precipitin technic yielded only qualitative information because of the incomplete precipita-

TABLE I. Total N (μg/ml) Precipitated by Homologous Casein, Whey and Milk per ml of Sera from the Sera of Male Rabbits Immunized with Weekly Injections of Homologous Milk Adjuvants.

Rabbit No.	Bleeding	Antigen added/ml of sera: Casein (μg N) 14	30	60	120	180	Whey (μg N) 20	40	80	100	160	Milk (dil.) 1-100	1-40	1-20	Hemagglut. titers†
7212	1/30/59*	8.4	0.6	5.2	2.4	4.8	—	4.4	11.2	13.2	7.2	13.2	21.6	22.0	2
"	6/24/59	36.4	57.6	41.2	41.6	33.6	8.4	—	3.6	14.4	6.0	68.8	235.6	180.8	64
7209	1/30/59	9.2	18.4	—	12.4	—	3.6	6.8	5.6	—	8.6	—	—	—	4
"	10/15/59	37.2	44.8	45.6	56.8	38.4	40.4	28.4	52.0	—	49.6	104.2	286.8	244.4	128

* First inj. given 1/31/59.

† Reciprocal of highest dilution of sera giving complete hemagglutination. Erythrocytes sensitized with homologous milk.

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TABLE II. Hemagglutinin Titers* of Sera from 5 Virgin Female Rabbits Immunized with Weekly Injections of Homologous Milk Adjuvant.

Rabbit No.	8/13/59†	8/14	9/2	9/23	10/12	11/25	1/12/60	2/25	5/16	6/21	7/1	7/14	7/21	7/30	Date of delivery
7681	2	8	8	4	32	8	128	32	64			16	16	8	7/15/60
7682	4	4	32	16	8	16	4	4							
7683	4	4	128	16	8	32	32	64	16						
7685	4	4	8	16	32	8	8	16	64						
7686	2	8	8	16	8	8	128	8	8	4	4		2		6/25/60

* Reciprocal of highest dilution giving complete hemagglutination. Erythrocytes sensitized with homologous milk.
† First inj. given 7/22/59.

tion of the antigens even in excess antibody. Hemagglutinins were measured by a modification of the Takatsy plate technic(18), employing a 1% solution of formalinized, tanned erythrocytes(19,20) sensitized with defatted rabbit milk (1:100 in saline). Five adult virgin female rabbits were injected with homologous milk-adjuvant and another 5 with homologous casein-adjuvant. The sera of both groups were tested for antibody by hemagglutination and precipitin tests at monthly intervals. Two of the female rabbits receiving milk and 4 receiving casein were then bred. Bleedings and breast biopsies were taken approximately 1 day, 1 week, 3 weeks and 5 weeks post partum. The tissue was fixed in 10% formalin and embedded in paraffin. Sections were stained with H & E and examined microscopically. Three does were injected with autologous milk-adjuvant. Two of these 3 were bled, bred and biopsied as described above. The sera of the pregnant animals were tested for antibody to whole milk by the hemagglutination technic. Control breast biopsies were also taken from 8 non-immunized does 1 to 22 days post partum.

Results. Homologous rabbit anti-milk antibody was demonstrated by precipitation and by hemagglutination in the sera of the 2 male rabbits immunized with milk-adjuvant (Table I). Precipitin tests were positive to casein, whey and defatted milk with serum from rabbit 7209 but positive only to casein and defatted milk with serum from rabbit 7212. Post immunization serum from rabbit 7209 formed 2 precipitin bands with whole milk in agar, while the serum from rabbit 7212 formed only one band with whole milk. Preimmunization sera failed to form any bands with whole milk in agar or to agglutinate erythrocytes sensitized with milk. Homologous anti-milk antibody was also demonstrated by hemagglutination and precipitation in the sera of the female rabbits immunized with either homologous milk in adjuvant (Table II) or homologous casein in adjuvant (Table III). Following delivery, 3 of the 6 rabbits immunized with homologous material showed what appeared to be a secondary rise in antibody titer. No circu-

TABLE III. Hemagglutinin Titers (HA)* and Precipitation (P)† of Sera from 5 Virgin Female Rabbits Immunized with Weekly Injections of Homologous Casein Adjuvant.

Rabbit No.	Date of bleeding‡ 8/13/59 (HA)	9/9/59 (HA)	9/23/59 (P)	9/23/59 (HA)	10/12/59 (P)	10/12/59 (HA)	11/20/59 (P)	11/20/59 (HA)	12/1/59 (HA)	12/15/59 (HA)	Date of delivery
7758	4	16	11.6	128	13.6	16	26.6	4	32	32	11/21/59
7759	4	64	8.8	8	11.2	128	7.6	32	4	32	11/24/59
7760	4	32	1.6	8	12.8	64	10.4	2	16	8	11/23/59
7762	4	64	2.0	16	13.2	16	29.6	16	32	8	11/23/59
7763	2	4	—	4	8.0	32	11.2	Died			—

* Reciprocal of highest dilution giving complete hemagglutination. Erythrocytes sensitized with homologous casein.

† Total μg N precipitated/ml of sera by addition of 22 μg casein N. The μg N precipitated by prebleeding sera (8/13/59) were subtracted from each value.

‡ First inj. given 8/14/59.

lating antibody could be demonstrated in the sera of the 3 rabbits immunized with autologous milk. Post partum breast biopsies of rabbits injected with either homologous or autologous material revealed no lesions that could be classified as "auto-allergic"(21). Some infiltration of plasma cells was observed between acini, but this infiltration was seen with equal frequency in control animals post partum.

Discussion. Following injection of 2 male rabbits with homologous milk incorporated in Freund's complete adjuvant, antibody to milk and casein appeared in the sera. The serum of one of the male rabbits also contained antibody to homologous milk whey. Female rabbits injected with homologous casein also formed circulating antibodies reacting with both whole defatted milk and casein. On the other hand, female rabbits immunized with autologous milk did not produce circulating antibody to autologous milk. Breast biopsies taken from lactating rabbits which had been previously injected with either homologous or autologous milk revealed no significant, consistent histologic abnormalities. This finding does not rule out the possibility that some forms of mastitis in humans might be "auto-allergic" in etiology; however, attempts to demonstrate circulating antibody in humans with plasma cell and chronic cystic mastitis also have been unrewarding(22). A suggestive case has been reported of a lactating mother who suffered from allergic reactions while weaning her child and had sever cutaneous reactions to her own mlik(23). This sensitivity could be passively transferred with her sera.

Summary. Injection of both male and female rabbits with homologous milk or casein in adjuvant resulted in formation of homologous anti-milk antibody demonstrated by hemagglutinin and precipitin technics. Injection of female rabbits with autologous milk did not result in formation of antibody to autologous milk. "Auto-allergic" breast lesions did not occur during lactation in females injected with either homologous or autologous milk.

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A Study of Lipoprotein Lipase Produced by Perfusion of Isolated Rabbit Heart with Heparin.* (26776)

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Intravenous administration of heparin increases a clearing factor in human and animal plasma. This clearance has been shown to be a result of the appearance in the serum of lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglyceride moiety of chylomicron and low density lipoprotein(1). Using an isolated rat heart, Crass demonstrated the appearance of LPL in the perfusing solution, which consisted of 5% serum and 3 to 10 mg % heparin in Ringer buffer (2). In this paper we are interested in the metabolism of transport of lipides relating to the problem of atherosclerosis. The mechanism of appearance of LPL in circulating solution in presence of heparin and serum was studied.

Method. The rabbits used were 8-month-old males weighing 1.5 to 2.0 kg with hearts of 3.5 to 5.0 g. Sodium heparin contains 1,000 U of heparin per ml with a concentration of about 10 mg/ml. A rabbit was anesthetized with ether and exsanguinated. Its heart was immediately isolated and washed well with bicarbonate buffer of pH 7.4 (Krebs-Ringer). Care was taken to avoid introduction of air into the circulating system. The aorta was connected to a glass tube and then by rubber tube to a pump (Sigma motor T-6). Rubber tubing was used to connect the pump to the polyethylene tube which ran to the accepting flask. Rubber contamination appearing during contact with the pump was filtered off through a glass tube filled with gauze. O_2 gas was bubbled through the liquid in the flask at a rate of 2 to 3 bubbles per second. Except in the pump, temperature of the circulating liquid was maintained at 30 to 31.5°C by keeping the entire perfusion system in a water bath of 33°C. With the pump set to deliver 10 to 12 ml/min, the isolated heart contracted well for 2 to 3 hours. The circulating liquid consisted of varying concentrations of heparin and serum (described in Table I) in bicarbonate buffer of Krebs-Ringer (pH 7.4). At the start of the experiment total volume of circulating solution was 120 ml. Eight ml of perfusing solution was taken from the flask at intervals of 20 min and immediately stored at 0°C until determination of LPL activity.

Determination of enzyme activity. Three ml of perfusion solution were incubated with 0.5 ml of 3% oil emulsion (coconut oil : glucose : Tween 60 = 15 : 5 : 0.5), 0.5 ml of

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TABLE I. Effect of Heparin and Serum on LPL Production.

Serum (%)→	5	10	50	—	5	5	—
Heparin (mg %)→	5	5	5	5	—	10	—
Time (min.)							
20	.025	.060	.065	—	.023	.023	—
40	.033	.090	.073	.015	.026	.043	.005
60	.130	.143	.105	.040	.040	.043	.010
80	.135	.150	.155	.050	.043	.047	.008
100	.128	.107	.166	.028	.033	.042	—
120	.030	.090	.083	.018	.020	.035	—

Time: Perfused time.
Values were increased optical density at 570 mμ after incubation for 1 hr in 5 ml reaction mixture with 1 ml of enzyme solution produced per 1 g of heart.
Mean variation was ± 15% with 5 times experiments at maximum enzyme activity.

10% albumin and 1.0 ml of M/10 ammonium chloride buffer containing M/25 $CaCl_2$ at 37°C(3). At 0, 1 and 2 hours of incubation 1 ml of the reaction mixture was put into a glass tube containing 0.5 ml of 10% trichloroacetic acid solution. Glycerol was determined in the deproteinized filtrate according to Korn's method(4), using chromotropic acid reagent.

Results. Effect of serum and heparin on LPL production. LPL activity in each perfusing solution was determined in the presence of various amounts of serum and heparin (Table I). Appreciably high enzyme activity was demonstrated in solutions of 5, 10 or 50% serum with 5 mg % heparin, whereas only slight activity was demonstrated with buffer alone. Addition of heparin or serum alone produced considerable LPL activity; however, higher activity was produced in the presence of both. A concentration of heparin as high as 10 mg % in addition to 5% serum inhibited the appearance of LPL, perhaps because of the inhibiting effect of heparin on LPL activity as reported *in vitro* experiment (I). The pH of the perfusion solution rose to 8.5 soon after the introduction of O_2 into the solution, due to CO_2 liberation. The experiment was, however, carried out with this pH, since the enzyme was not inactivated.

TABLE II. Effect of Heat on Serum to Liberate LPL Activity.

Time (min.)	Control	Serum heated at: 50°C for 1 hr	70°C for 2 min.
20	.027	.030	.035
40	.042	.033	.068
60	.107	.115	.073
80	.133	.142	.099
100	.116	.073	.022
120	.038	.030	.010

Time: Perfused time.
Values are same as Table I.

Effect of heat and dialysis of serum. Serum, 5% in bicarbonate buffer, was heated at 50°C for 60 min and at 70°C for 2 min. Perfusion was carried out with each solution, after addition of 5 mg % heparin (Table II). The serum in a visking cellulose tube was dialyzed by ultrafiltration technic(5) under decreased pressure (60 to 65 mm Hg) at room temperature of 10 to 15°C. The dialyzable serum fraction was computed to be 5% of the original serum. After the addition of 5 mg % heparin, this fraction was perfused; however, LPL did not appear (Table III). The non-dialyzable fraction of serum was prepared by dialyzing serum against distilled water or bicarbonate buffer of pH 7.4 at 5°C overnight. The activity produced by this fraction, though appreciably decreased in quantity, was similar to that produced with untreated serum. (Table III).

Effect of phosphate buffer. Heparin and serum were added in phosphate Ringer buffer (pH 7.4 or 8.5); other conditions for perfusion were the same as with bicarbonate buffer. There was no change in pH during perfusion. LPL activity was not demonstrated in circulating solution of either buffer. Since phosphate ion inhibits LPL activity *in vitro* (1), the results obtained might be due to the inhibiting effect of phosphate on the LPL produced; however, it is possible that LPL is not produced in presence of phosphate.

Effect of inhibitors on LPL. A perfusion

TABLE III. Effect of Dialysis of Serum on LPL Liberating Activity.

Time (min.)	I Control*	II Heparin†	III Heparin 5% serum dialyzed	IV Heparin 5% dialyzable serum fraction
20	.027	—	.030	.012
40	.042	.015	.050	.020
60	.107	.040	.085	.042
80	.133	.050	.118	.043
100	.116	.028	.080	.020
120	.038	.018	.050	.020

* Control solution contained 5% serum and 5 mg % heparin.
† Heparin: 5 mg % used.
Values are same as Table I.

solution obtained from 80 min perfusion in the presence of 10% serum and 5 mg % heparin under conditions producing the highest activity was used as the LPL solution (Table I). The enzyme had the same properties as LPL of adipose tissue(4) and plasma(3) in its behavior in presence of its inhibitors. There was evidence, however, of other esterases which were not inhibited by protamine sulfate or by higher concentrations of NaCl or $CaCl_2$ (Table IV).

Optimum pH of LPL. The enzyme solution used in the above experiment was adjusted to pHs of 7.2, 7.6, 8.0 and 8.4, with addition of M/40 aqueous ammonium solution of M/40 HCl. Buffer, substrate and albumin were adjusted to the corresponding pH values. The optimum pH of enzyme activity was shown to be 8.2, which is similar to the LPL of adipose tissue (Fig. 1).

Thermostability of LPL. Each enzyme solution described above was heated to the temperatures noted in Table V and its enzymatic activity studied (Table V).

TABLE IV. Effect of Inhibitors on LPL.

Inhibitors	Conc.	O.D.$_{570\ m\mu}$	Activity, %
NaCl	1 M	.022	35
"	.5 M	.040	63
$CaCl_2$	.2 M	.022	35
"	.1 M	.022	35
Na-protamine sulfate	200 γ/ml	.032	57
Idem	100 "	.042	67
Control	—	.063	100

O.D.$_{570\ m\mu}$: Increased optical density in 0.2 ml of reaction mixture, after 1 hr incubation.

LPL activity of intact and perfused rabbit heart. Fresh heart tissue was glass homogenized with 3 volumes of M/10 ammonium chloride buffer (pH 8.5) at 0°C. The homogenate was centrifuged at 0°C after

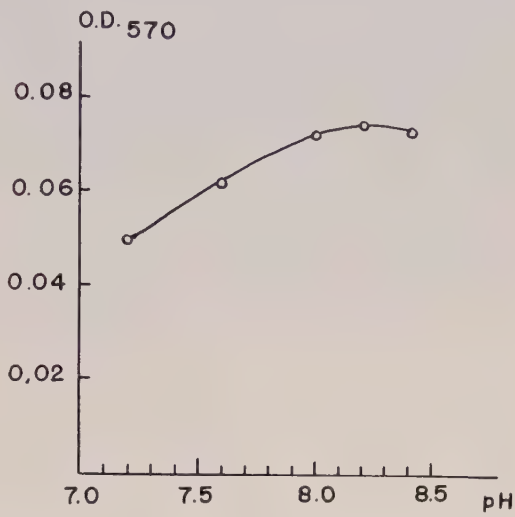

FIG. 1. Optimum pH. O.D.$_{570}$: Increased optical density at 570 mμ in 0.2 ml of reaction mixture, after 1 hr incubation.

standing for 30 min at 0°C. After addition of 2 ml of distilled water to make a final volume of 5 ml, the supernatant of 1.0 ml was used for activity assay, under the same conditions described elsewhere.

TABLE V. Thermostability of LPL.

Temperature	Period	O.D.$_{570\ m\mu}$	%
37°	30 min.	.068	87
"	60 "	.053	68
50°	30 sec.	.062	79
"	1 min.	.040	51
"	2 "	.011	24
70°	30 sec.	.016	21
Control		.078	100

O.D.$_{570\ m\mu}$: Increased optical density in 0.2 ml of reaction mixture, after 1 hr incubation.

Ammonium buffer extractable LPL activities of the intact and perfused hearts were compared. With intact hearts, the increase in optical density at 570 mμ due to glycerol in 0.2 ml of reaction mixture was shown to be 0.090 as a mean value ($\pm$ 0.015), whereas after the perfusion experiment, LPL activity was completely lost (after 100 min of perfu-

sion). Total activity appearing in the perfusion solution was calculated from Table I; the LPL activity obtained from the perfusion solution was about 1.5 times that obtained from the ammonium extractable LPL of heart.

Summary. With a perfused, isolated rabbit heart LPL activity appeared in the perfusion solution of bicarbonate buffer in the presence of 5 mg % heparin and 5 to 10% serum. However, under the same conditions, LPL did not appear with phosphate buffer. The LPL produced was inhibited by protamine sulfate, $CaCl_2$ and NaCl. Optimum pH of enzymatic activity was at pH 8.2, and 80% of the activity was lost by heating at 50°C for 2 min and 70°C for 30 sec. After perfusion, LPL activity in the heart disappeared. Total activity appearing in the circulating solution was nearly equal to total activity of heart tissue which was extractable with ammonium buffer solution.

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Conversion of Mevalonic Acid-2-C^{14} to Biliary Cholesterol and Cholanic Acids in the Guinea Pig.* (26777)

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Since mevalonic acid was discovered(1) numerous studies have been conducted to show the conversion *in vitro* of this compound to cholesterol(2,3). Important intermediates in the metabolic pathway appear to be phosphomevalonic acids, pyrophosphates of isopentenyl and dimethylallyl alcohol, geraniol, farnesol and nerolidol, as well as squalene and lanosterol(3). Cholesterol, in turn, is the major precursor of bile acids. In comparison to many studies on conversion of mevalonic acid to cholesterol *in vitro*, few investigations have been reported on *in vivo* conversion to cholesterol(4) or to bile acids. The present study concerns *in vivo* conversion of mevalonic acid-2-C^{14} to labeled cholesterol and bile acids in guinea pig.

Materials ad methods. Each of 25 guinea pigs of either sex, weighing 140-180 g, received, intravenously, 3.26 μc (0.772 mg) mevalonic acid-2-C^{14} in 0.10 ml 0.15 molar NaCl. Four hours later, all animals were sacrificed and the contents of their gall bladders pooled. Bile acids and cholesterol were then isolated, identified and the C^{14} content determined by the following procedures.

The 4.20 ml of pooled bile were hydrolyzed in 1 N NaOH for 5 hours at 110°C, extracted with ethyl ether and further analyzed on a column of hydrophobic kieselguhr using chloroform:heptane (9:1) as stationary phase and 58% aqueous methanol as mobile phase(5). Aliquots of the original sample of pooled biles, of the ether extract, and of the fractions of the effluent from the column were applied to aluminum planchets and their C^{14} content determined by a windowless flow counter with accumulation of sufficient counts for 1% standard error.

Results. The pooled bile contained 2.2% of injected C^{14}. Of this activity, 99.3% was recovered in ether extract after hydrolysis. Most of the C^{14} applied to the column was found in the fractions having the mobility of 3α, 7α-dihydroxycholanic (59.7%) and 3α-hydroxy, 7-ketocholanic acid (28.5%). After collection of 150 ml of effluent, the column was eluted with $CHCl_3$, and 11.8% of C^{14}

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was recovered, representing the non-mobile "neutral sterols."

The 2 labeled bile acids were mixed with corresponding unlabeled acids, obtained from guinea pig bile and identified by column (5) and paper chromatography(6), infra red spectra, spectra in conc. H_2SO_4, specific rotation and melting point. No depression of melting points was observed and C^{14}/mg remained constant after several recrystallizations. To identify the sterols in $CHCl_3$ eluate of the column, digitonin treatment(7) was performed. After the digitonide was split with pyridine, 91% of C^{14} (10.7% of total activity applied to the column) was recovered. This labeled material was mixed with authentic unlabeled cholesterol and brominized(8). Following splitting of dibromo compound, all activity was recovered as cholesterol. C^{14}/mg remained constant after several recrystallizations and the melting point was not depressed.

Comments. The results of the present study demonstrated that labeled cholesterol and bile acids appeared in bile of the guinea pig soon after intravenous administration of mevalonic acid-2-C^{14}. Gould and Popják(4) recovered approximately half of the injected C^{14} from the urine of the rats within 4 hours following intraperitoneal administration of mevalonic lactone-2-C^{14}, thus supporting the suggestion of Tavormina and coworkers(2) that only one enantiomorph of mevalonic acid is utilized for biosynthesis of cholesterol, the unnatural (−)-isomer being excreted in the urine(9). Urinary recovery of 48.7% of C^{14} after intravenous injection of mevalonic acid-2-C^{14} in guinea pig confirms the above observations in the rat.

Since conversion of cholesterol to bile acids has been established in many animals as well as the guinea pig, it would appear that mevalonic acid may have been converted first to cholesterol and thence to bile acids. According to the Woodward-Bloch proposal for squalene cyclization to sterol(10) and the positions of incorporation of C^{14} from mevalonic acid-2-C^{14} into cholesterol(3), one C^{14} atom per molecule or 20% of the isotope is lost in conversion of cholesterol to bile acids. Guinea pigs with continuous enterohepatic cycling for 4 hours after intravenous administration of cholesterol-4-C^{14} had 1.7% of injected C^{14} per ml of bile(11). In the present study, after a comparable period of enterohepatic cycling, the guinea pigs excreted in bile 4.4% of C^{14} from mevalonic acid not excreted in urine. Adjusting this to the 4.2 volume and by the 20% loss in conversion of cholesterol to bile acids, one arrives at a value of approximately 1.3% injected C^{14}/ml bile. The similar values for biliary excretion following mevalonic acid and cholesterol suggest that most of the mevalonic acid not excreted in urine entered the cholesterol pool as such. This ratio of conversion of mevalonic acid-2-C^{14} to cholesterol is similar to the observation in the mouse by Gould and Popják(4).

The 2 labeled bile acids isolated in this study, 3α, 7α-dihydroxycholanic (chenodeoxycholic) and 3α-hydroxy, 7-ketocholanic (7-ketolithocholic) acid, the only bile acids present in the bile of immature guinea pigs (12), appear normally in approximately the same ratio as observed in the present investigation.

Summary. After intravenous administration of mevalonic acid-2-C^{14} the labeled constituents of guinea pig bile were cholesterol, 3α, 7α-dihydroxycholanic (chenodeoxycholic) and 3α-hydroxy, 7-ketocholanic (7-ketolithocholic) acid. The relative amount of C^{14} in these compounds suggested that most of the C^{14} not excreted in urine entered the cholesterol pool as such.

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Effects of Controlled Acute Hemorrhage on Myocardial Contractile Force. (26778)

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The concept that cardiogenic factors are contributory to death from shock has been well documented since the early observations of Werle, Cosby and Wiggers(1). These investigators showed that pulsus alternans, progressive bradycardia, and an elevated right atrial pressure are terminal features of hemorrhagic shock. Pathologic study of dog hearts following hemorrhagic shock(2) has suggested that myocardial ischemia is the underlying cause of the cardiac depression. Further support is lent to this view by studies of coronary blood flow and myocardial oxygen consumption(3,4,5,6) which demonstrated that flow is decreased in spite of coronary vasodilatation and that myocardial oxygen consumption is reduced even though the extraction of oxygen from coronary arterial blood is increased. That a net decrease in cardiac efficiency is associated with shock has been generally agreed upon, but some investigators feel that the observed reduction in coronary flow is insufficient evidence that cardiac failure contributes to the progression of hemorrhagic shock and have suggested that other properties of the myocardium be evaluated to elucidate further the mechanics of adjustment to the shock state(7). In the present study myocardial contractile force was measured during acute hemorrhage and onset and extent of myocardial depression assessed by this means.

Methods. Adult mongrel dogs weighing 9-14 kg and apparently in good health were anesthetized with thiopental (30 mg/kg) and respiration was maintained through a cuffed endotracheal tube attached to a piston-type respirator. Central aortic pressure was obtained by cannulation of one femoral artery and the opposite femoral artery was cannulated and connected to an elevated calibrated reservoir. A Walton-Brodie strain gauge arch(8) was sutured to the anterior surface of the right ventricle after the right chest had been opened. The segment of myocardium between the points of attachment of the arch was stretched by at least 50% of its initial length so that any change in fiber length would not affect the measurement of contractile force(9). Pressures measured by means of Statham transducers were recorded continuously, along with myocardial contractile force, on a direct-writing polygraph. The animals were given heparin intravenously (2 mg/kg) and after a control observation had been made they were allowed to bleed into the reservoir at a steady rate. Eight dogs were bled at a rate which lowered mean aortic pressure to 50 mm Hg within 2 minutes; 4 dogs were bled to this level of pressure in 5 minutes and another 4 dogs were bled to this pressure in 10 minutes. Blood pressure was maintained at or below 50 mm Hg by continued withdrawal of blood as necessary during the period of observation and the animals were then sacrificed.

An additional group of 4 dogs was studied utilizing total right heart bypass. Cannulae were inserted into the superior and inferior vena cavae and a third cannula was inserted into the right ventricle to return coronary blood and provide total ventricular decompression. The venous return from these cannulae was collected in a reservoir and re-

turned to a branch of the pulmonary artery by a Sigmamotor pump. Systemic pressure was acutely reduced by decreasing rate of flow into the pulmonary artery, simulating acute bleeding. Arterial pressure was reduced to 40-50 mm Hg within 2 minutes and changes in right ventricular contractile force were recorded.

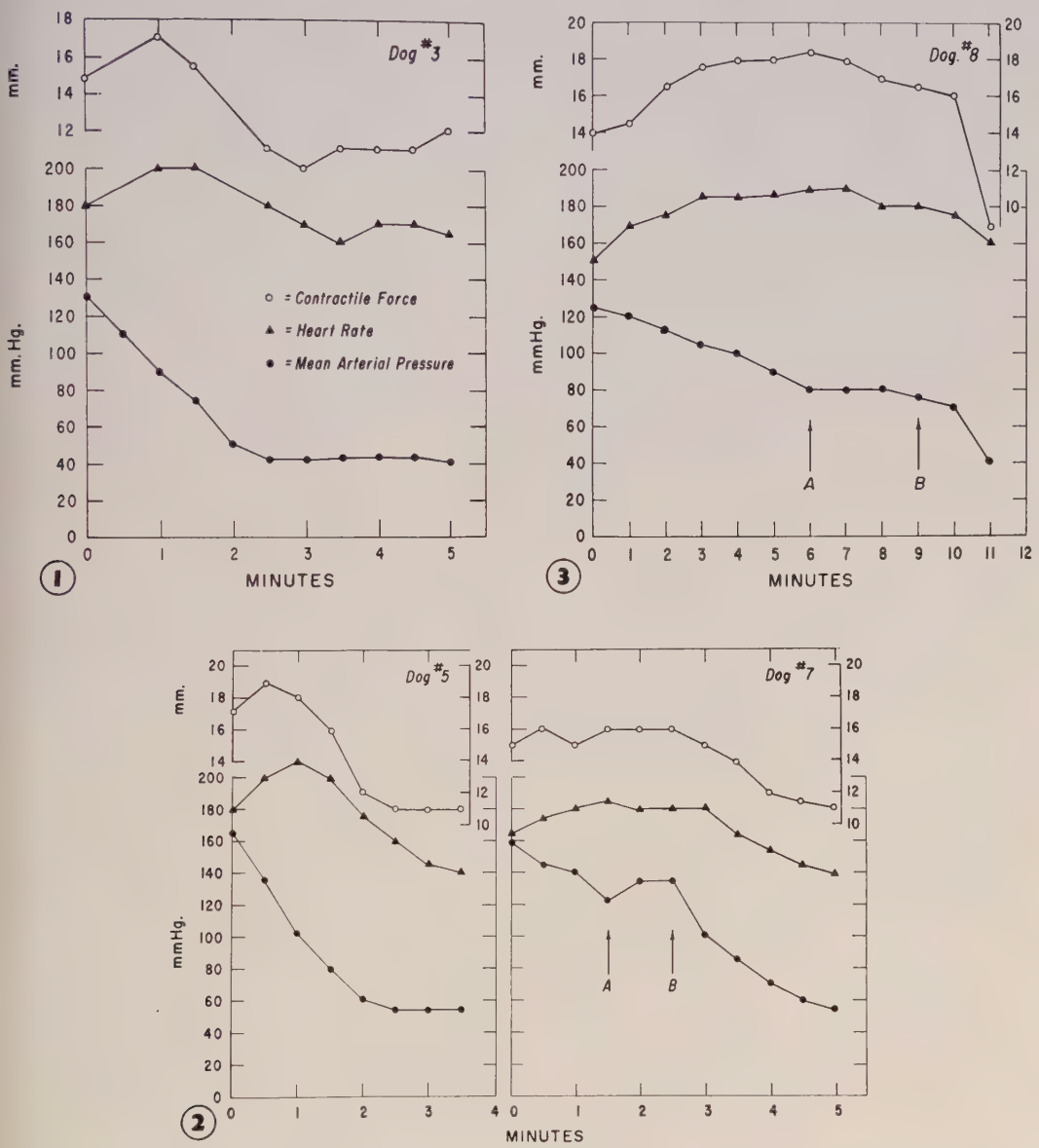

FIG. 1. Effects of acute hemorrhage on heart rate, myocardial contractile force and mean arterial pressure of a normal dog. Mean arterial pressure was lowered to 40 mm Hg within 2 min. of onset of bleeding.

FIG. 2. Effects of controlled acute hemorrhage on heart rate (▲—▲), myocardial contractile force (○—○) and mean arterial pressure (●—●) in 2 normal dogs. A mean arterial pressure of 50 mm Hg was attained within 2 min. in Dog #5 and within 5 min. in Dog #7. At point A in Dog #7 bleeding was stopped for 1 min., and bleeding was resumed at point B.

FIG. 3. Effects of acute hemorrhage on myocardial contractile force (○—○), heart rate (▲—▲) and mean arterial pressures (●—●). The dog was bled over a 6-min. period to a pressure of 80 mm Hg (point A). Bleeding was discontinued for 3 min. and then resumed (point B).

Results. The cardiovascular responses of a normal dog to sudden acute hemorrhage are shown in Fig. 1. With onset of bleeding there was an increase in heart rate and contractile force, but depression of both occurred when mean aortic pressure fell below 90 mm Hg. The decrease in contractile force preceded the fall in rate. Fig. 2 (dog #5) illustrates the findings in another experiment in which hemorrhage at approximately the same rate was induced; a decrease in contractile force occurred while heart rate was still increasing. Subseqeunt depression in both heart rate and contractile force were again noted when mean arterial pressure was lowered to 100 mm Hg. In the second experiment in Fig. 2 (dog #7) bleeding was stopped when arterial pressure reached 120 mm Hg (point A) and both rate and contractile force stabilized. When bleeding was resumed one minute later (point B) decreases in contractile force and rate were observed as arterial pressure fell to 100 mm Hg. In all animals bled to a mean arterial pressure of 50 mm Hg within 2 or 5 minutes an average initial increase in contractile force of 13.7% was noted. Contractile force and heart rate began to decrease at an average mean pressure of 95 mm Hg and when the pressure had fallen to 50 mm Hg, contractile force averaged 28.3% less than control levels. Measurements of right ventricular end-diastolic pressures in 4 of the dogs bled acutely showed no significant change during the period of observation.

The effects of a slower rate of bleeding are shown in Fig. 3. In this dog the gradual decline in arterial pressure was accompanied by a marked increase in heart rate and contractile force. When bleeding was stopped at a mean pressure of 80 mm Hg (point A) a gradual decrease in both contractile force and heart rate occurred. When bleeding was resumed 3 minutes later (point B) a more rapid decline in both modalities was observed. Observations in all 4 dogs bled to 50 mm Hg during a 10 minute period showed that average initial increase in contractile force was 27.6%. Decreases in contractile force and heart rate were noted at an average mean pressure of 86 mm Hg; at the end of the 10 minute observation period contractile force averaged 37.7% less than control levels.

In the dogs subjected to right heart bypass, the effects of acute hypotension on myocardial contractile force were identical to the effects of acute bleeding. Decreases in heart rate and contractile force occurred at an average mean pressure of 85 mm Hg and average depression in contractile force at the end of 5 minutes of hypotension was 47.8%. At the end of the hypotensive period, rate of perfusion was increased sufficiently to return arterial pressure to control levels; the contractile force showed a corresponding return to an average of 105% of control.

Discussion. The cardiovascular response to hemorrhagic shock in dogs has been studied in most cases after shock levels of arterial pressure had been induced. At this level of circulatory depression, it has been shown that there is coronary vasodilatation(6), decreased coronary flow(6), elevated atrial pressure(3), decreased stroke output(10), and increased oxygen extraction from coronary blood(5). From investigations made during induction of shock it has been shown that a metabolic conversion occurs from the aerobic phase of the Krebs cycle to an anaerobic phase with increased levels of pyruvate and lactate in coronary venous blood(4). It has also been demonstrated by electropolarographic technics that a decline in myocardial oxygen availability in the tissues occurs in proportion to the decline in arterial pressure induced by rapid hemorrhage(12). The changes in contractile force noted in the present study no doubt result from the metabolic and hemodynamic alterations above. In every animal, regardless of rate of bleeding, a decrease in contractile force occurred when mean arterial pressure fell to 85-95 mm Hg, and shortly thereafter was accompanied by a fall in heart rate.

In the animals subjected to right heart bypass, identical depression of heart rate and contractile force was noted during hypotension. As arterial pressure was increased to normal, heart rate and contractile force returned to control levels. This constant relationship of contractile force to arterial pressure, and the coincident decrease in heart

rate, support the assumption that the changes are due to myocardial depression. The most logical explanation for the depression of contractile force and heart rate below a mean arterial pressure of 90 mm Hg is that this represents a critical coronary perfusion pressure. With profound hypotension the role of coronary insufficiency in the depression of ventricular function has been previously shown(11), but the present results indicate that neither prolonged nor severe circulatory depression is necessary for the occurrence of myocardial depression in response to hemorrhage. In a study of dogs with totally denervated hearts* neither augmentation nor depression of heart rate and myocardial contractile force occurred in response to acute hemorrhage. These findings suggest that in addition neurogenic factors influence the myocardial response to rapid blood loss.

Summary. The changes in myocardial contractile force and heart rate which accompanied acute bleeding were studied in normal dogs. Increases in contractile force and heart rate were noted in response to hemorrhage until mean arterial pressure fell to less than 90 mm Hg. Below this level of pressure depression of both modalities began and continued as pressure was further lowered. These findings indicate that myocardial depression occurs at an early stage in acute bleeding and probably results from a critical diminution in coronary perfusion pressure.

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Partial Purification of Cattle Serum Transferrin Using Rivanol.* (26779)

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Chemical studies of genetic differences in proteins are invaluable in understanding the mechanism of gene action. Inherited variations in the serum proteins of many animal species can be used to study gene-controlled protein specificity(1). Transferrin, also known as siderophilin or β-metal combining globulin, appears to be a very promising subject for such a study, since inherited variations in this protein have been demonstrated in many animal species(2-5), and much is known about its chemical properties(6).

Transferrin of cattle serum can be resolved into at least 4 components by starch gel electrophoresis(7,8). The mobility of all of these are affected by a change in the transferrin locus. Five alleles have been described so far, designated β^A, β^B, β^D, β^F, β^E, in order of decreasing mobility of the corresponding transferrin(9). Different breeds of cat-

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tle have different frequencies of these alleles (9,10).

Transferrin is usually isolated by fractionating serum with ethanol or ammonium sulfate(11-13). Boettcher *et al.*(14) have devised a simpler approach. Rivanol (2-ethoxy 6,9-diaminoacridine lactate) is added directly to human serum to precipitate albumin and most of the α-globulin fraction. Transferrin has been purified and crystallized from this supernatant(15). In our work, transferrin from cattle serum has been partially purified using rivanol. Our ultimate goal is to prepare cattle transferrin pure enough for chemical studies of the phenotypic differences. In addition, we wish to determine the nature of the apparent multiplicity of this protein.

Materials and methods. Rivanol was obtained from the Special Chemical Department of Winthrop Laboratories under the trade name "Ethodin." Fractionation was carried out as follows: Serum was saturated with respect to iron by addition of 12 μg iron (as ferrous ammonium sulfate) per ml serum. The mixture was kept at 5°C for one hour. A predetermined volume (see below) of 0.4% aqueous rivanol was then added to one volume serum. The final pH was adjusted to 8.5-8.6 with 0.1 *N* sodium hydroxide and the mixture kept at 5°C for one hour. The precipitate was removed by low speed centrifugation, and dissolved in 0.1 M acetate buffer, pH 4.7. The supernatant was treated with Norite or dialyzed to remove rivanol.

Electrophoresis of all fractions was carried out in vertical starch gels(16) using borate buffer, pH 8.6. Some of the fractions were also analyzed by paper electrophoresis using an E-C pressure-plate electrophoresis apparatus. The buffer used contained 58.8 g sodium barbital, 23.4 g anhydrous sodium acetate, 3 l distilled water, and 2.5 ml glacial acetic acid. The final pH was 8.6. After electrophoresis in starch gel or on paper, the proteins were stained with Amido Black. Six μl serum were used for analysis on paper and about 0.1 ml for starch gel.

Results. One-volume samples of serum were treated with increasing volumes of 0.4% rivanol to determine the ratio of rivanol to serum that would give maximum purification of transferrin. After addition of the appropriate amount of rivanol, the precipitates were removed by centrifugation and the supernatants analyzed by starch gel electrophoresis. The rivanol migrated towards the cathode and off the gel completely. No correction was made for the dilution effect of the rivanol solution added. The results of this first experiment are shown in Fig. 1. Addition of an equal volume of 0.4% rivanol to serum did not affect the pattern on starch gel appreciably, while a 2:1 ratio of rivanol to serum removed a large amount, but not all, of the albumin and some of the slow α-globulin. At a ratio of 3:1 there was no albumin and very little if any slow α-globulin visible on the gel. At this ratio, the transferrin bands were clearly visible and apparently unaffected by the rivanol treatment, as compared to the untreated serum. At ratios above 3:1, the transferrin bands became faint, but were still clearly visible. From these results it was concluded that the optimum ratio of rivanol to serum was 3:1, and this ratio was used in all further experiments.

In the next experiment, serum from the same cow, (BF 486, Holstein-Fresian Breed, type β^D/β^D) was treated with rivanol, and after removal of the rivanol, the supernatant was lyophilized and redissolved in distilled water to the same volume as the original serum. Likewise, the precipitate was reconstituted using 0.1 *M* acetate buffer at pH 4.7. A considerable amount fo protein in this fraction failed to dissolve so the sample used for electrophoresis was heterogeneous. Only transferrin and γ-globulin were clearly visible in the supernatant fraction. As expected, the insoluble fraction contained albumin and slow α-globulin, but only trace amounts of transferrin and γ-globulin.

The fractions of serum obtained after treatment with rivanol were studied also by paper electrophoresis. The insoluble fraction contained considerable amounts of β-globulin, even though only a small amount of transferrin was present. Also, a considerable amount of insoluble protein remained at the origin in this fraction. The evidence from both paper and starch gel electrophoresis in-

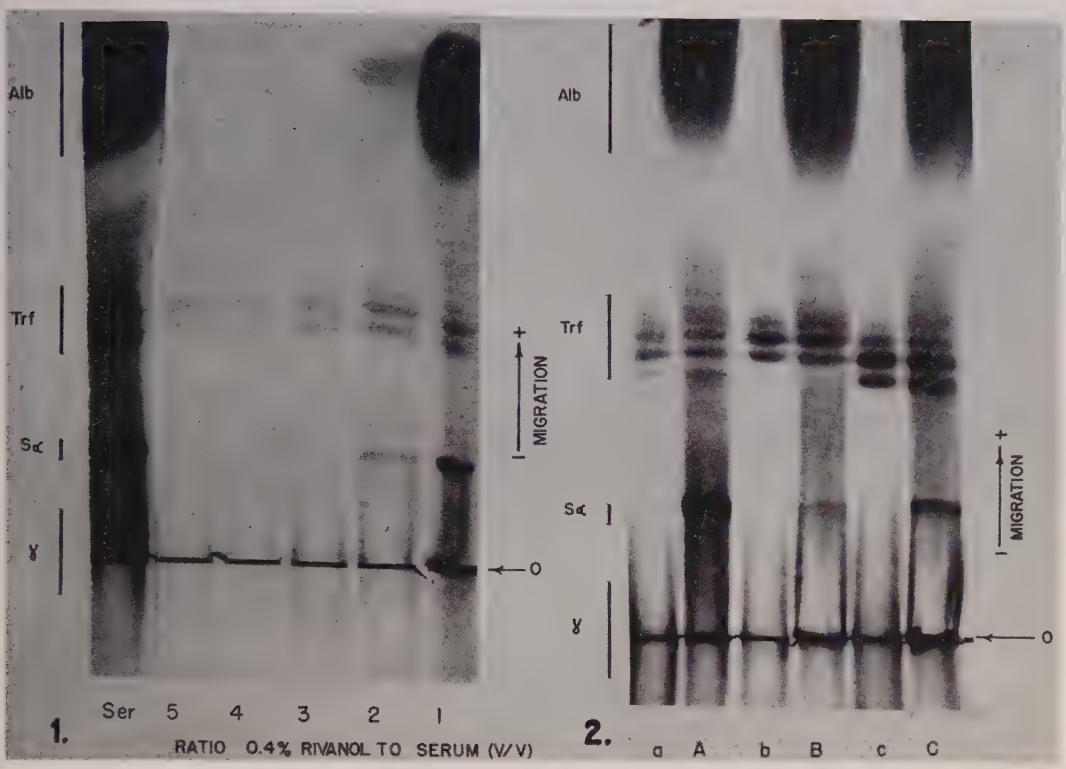

FIG. 1. Starch gel electrophoresis patterns of fractions of cattle serum after addition of varying amounts of rivanol solution. Ser = untreated serum. Samples 1-5 = soluble fractions after addition of 1-5 volumes of 0.4% rivanol to one volume serum. (Cow BF486, type β^D/β^D.) O = origin; γ = γ-globulin; Sα = slow α-globulin; Trf = transferrin; Alb = albumin.

FIG. 2. Starch gel electrophoresis patterns of serum from cows of 3 different transferrin phenotypes and corresponding supernatant fractions of sera after treatment with 0.4% rivanol. A = serum of type β^A/β^D; a = supernatant fraction of A; B = serum of type β^A/β^A; b = supernatant fraction of B; C = serum of type β^D/β^D; c = supernatant fraction of C. Other symbols as in Fig. 1.

dicates that treatment of cattle serum with 0.4% rivanol removes albumin and the α-globulin fraction.

Serum from each of 3 cows with phenotypically distinct transferrin types was treated with rivanol to see if the transferrin behaved similarly. As shown in Fig. 2, the supernatant fractions of all 3 cattle sera contained transferrin with the same patterns and mobilities as the untreated sera.

The genetically controlled variants in the serum β-globulin of humans and cattle were first shown to be transferrin by autoradiographs using iron-59(3). We have confirmed this for cattle using our partially purified transferrin preparations.

Discussion. Our results with rivanol fractionation of cattle serum closely parallel those of other workers using human serum (14,17). At ratios of 3:1 (v/v) 0.4% rivanol to serum, the albumin and α-globulin fractions of cattle serum are almost completely precipitated, leaving transferrin and γ-globulin in solution. Although cattle transferrin can be resolved into at least 4 bands by starch gel electrophoresis, treatment of serum with rivanol does not differentially affect the bands. In this respect, transferrin behaves as a unit.

Nothing is as yet known about the chemical basis of the differences observed between individuals of different transferrin phenotypes. Differences in mobility have been demonstrated by paper electrophoresis for the products of different transferrin alleles in both human and mouse serum(2,4). Human and porcine transferrin have been crystallized and found to contain considerable amounts

of sialic acid and hexosamine(6), so that the genetically controlled differences may involve either amino acids or carbohydrates. The nature of the multiplicity of transferrin from cows homozygous at the transferrin locus also remains an unsolved problem. It is possible that the bands are composed of the same protein with different numbers of sialic acid moieties attached. It has been found that treatment of human transferrin with neuraminidase results in the appearance of 5 bands whose intensity varies with the concentration of the enzyme(18,19). Direct evidence bearing on these questions, however, must await further purification of these proteins.

Summary. A ratio of 3 volumes 0.4% rivanol to one volume serum was used to partially purify cattle transferrin. After treatment, the supernatant fraction contained predominantly transferrin and γ-globulin. No albumin was detectable by starch gel or paper electrophoresis. Fractionation of serum from cattle of phenotypically different transferrin types yielded transferrin with the same pattern and mobility in starch gel electrophoresis as in the original serum.

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Pharmacological Studies with Polythiazide, a New Diuretic and Antihypertensive Agent.* (26780)

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The search for orally effective and safer diuretic agents led to the introduction of benzthiadiazines as a new class of diuretics (1,2).

Recently attempts have been made to develop more potent benzthiadiazines with a

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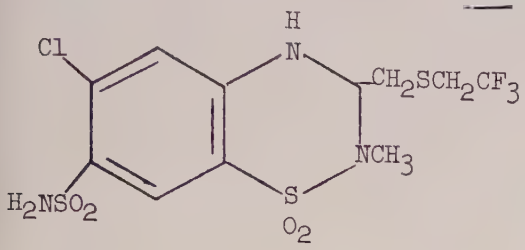
greater specificity in their natriuretic action, lesser effect on excretion of potassium or on glomerular filtration rate. Such efforts culminated recently in synthesis and development of polythiazide, 2-methyl-3-(β,β,β-trifluoroethyl - thiomethyl) - 6 - chloro - 7 - sulfamyl-3,4-dihydro-1,2,4 benzothiadizine, 1, 1 dioxide.



Material and methods. Polythiazide occurs as a white crystalline substance with a molecular weight of 439.9 and a melting point 214-215°C. The compound is insoluble in water but dissolves readily in alkaline solution.

To study the diuretic activity of polythiazide in rats the method of Lipschitz *et al.*(3) was used. Each animal was placed in a separate metabolism cage for a 5-hr period. Concentrations of Na^+, K^+ and Cl^- were determined in each collected urine sample. Results were expressed as $\frac{T\text{-}C}{5\text{ hr}}$ where T is urine volume or electrolyte concentration in the urine sample collected from a treated animal and C is average control value for 8 animals. A total of 112 rats was used in this study.

The saluretic activity of polythiazide was evaluated in 8 trained female dogs during water diuresis. To produce water diuresis the animals received orally a prime of 40 ml of water per kg of body weight and 20 ml/kg hourly thereafter. The dogs were trained to remain without anesthesia or restraint on trough shaped boards for 6-7 hours. Urine was collected by retention catheter continuously at hourly or at 10-30 minute intervals. Four experiments were made at each dose level of each drug. Polythiazide was given orally or intravenously. The increase in sodium or chloride concentrations in the urine was used to measure the saluretic effectiveness of the drug.

To study the duration of action of polythiazide over a 24 hour period 6 female mongrel dogs were deprived of food and water during the night prior to and throughout the experimental period. The animals received drugs orally. Urine samples were collected by repeated catheterizations. The effects of the diuretics were expressed as T-C, where T is average urine volume or amount of electrolytes excreted by 6 dogs after treatment and C is average control value for the same dogs.

Renal clearance experiments were performed on dogs lightly anesthetized with sodium pentobarbital, 25 mg/kg I.V. Isotonic dextrose or NaCl solutions were infused at the rate of 0.4 ml/min/kg for 2½ hours prior to and during the experiment.

Experimental metabolic acidosis was produced in 4 female mongrel dogs by dietary supplement of ammonium chloride, 0.5 g/kg/day for 5 days prior to the test. On the test day 5% dextrose solution was infused at 0.4 ml/min/kg for 2½ hours prior to and throughout the experiment. Experimental metabolic alkalosis was produced in female mongrel dogs anesthetized with sodium pentobarbital (25 mg/kg I.V.) by infusion of a solution containing 0.075M $NaHCO_3$ and 2½% dextrose (1 ml/kg/min for 10 minutes and 0.4 ml/min/kg for one hour).

Sodium and potassium in urine and plasma were determined with the Process Instruments flamephotometer. Urinary chlorides were determined by a method of Sendroy(4) and chlorides in plasma by the method of Schales and Schales(5). Determinations of total CO_2 in urine and plasma and calculation of bicarbonate concentration were done according to Peters and Van Slyke(6). Determinations of creatinine were made by the Folin method(7) and PAH was determined according to Smith *et al.*(8). Determinations of ammonium concentration in urine were done by the Conway method(9).

The changing pH method of Philpot and Philpot(10) was employed for estimation of carbonic anhydrase inhibitory activity as previously described(11).

To study the effect of chronic administration of polythiazide on blood pressure of hypertensive dogs systolic and diastolic pres-

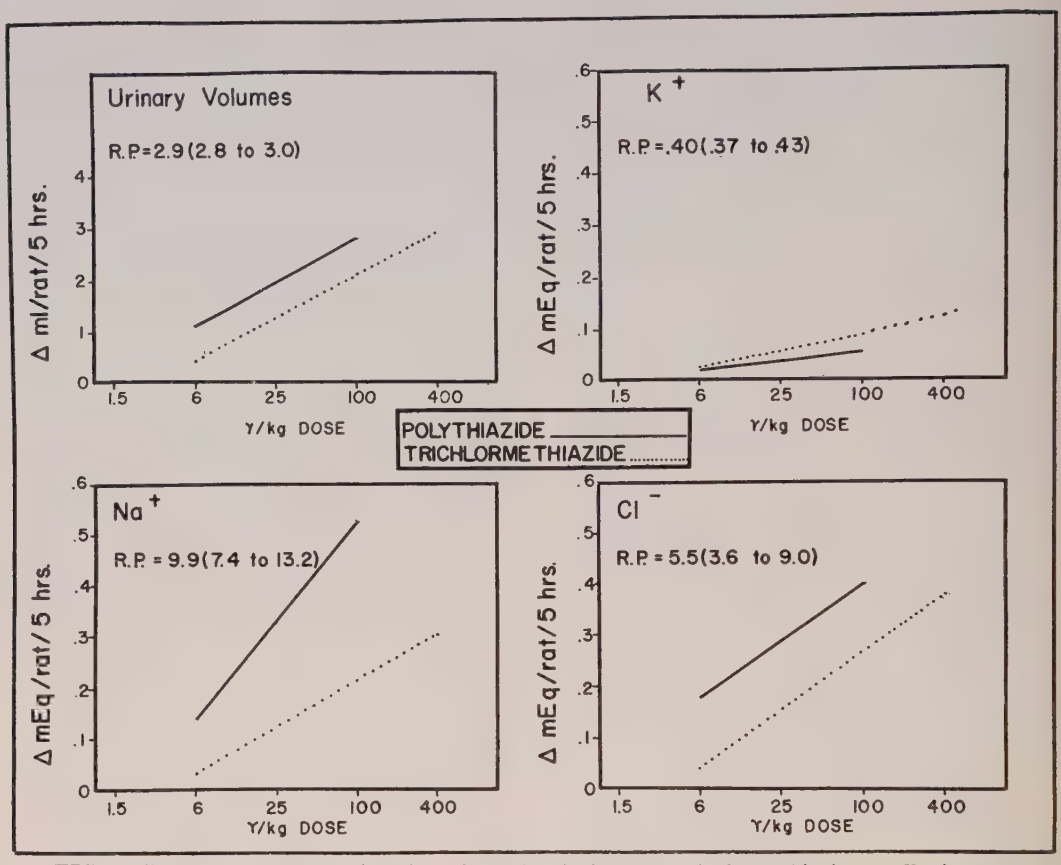


FIG. 1. Dose-response regression lines for polythiazide and trichlormethiazide orally in rats. Effects on urinary flow, Na^+, K^+ and Cl^- excretion are expressed as differences in excretion between an animal under test and an avg value for 8 control rats. Regression lines were calculated on the basis of 56 observations for polythiazide and 72 observations for trichlormethiazide. R.P. = Relative potencies with 95% confidence limits. Potency of trichlormethiazide is considered equal to 1.

sures were measured in 8 hypertensive unanesthetized dogs by the method of Prioli and Winbury(12). Three types of hypertensive dogs were used: (a) 4 renal hypertensive dogs prepared by the method of Goldblatt *et al.*(13); (b) 2 neurogenic hypertensive dogs prepared by the method of Grimson (14); and (c) 2 dogs exhibiting a profound and sustained spontaneous hypertension. The dogs were randomly divided into 2 groups of 4 dogs per group. One group received daily oral dose of 400 μg/kg of polythiazide (by stomach tube) for 5 days; the other group served as controls. Systolic and diastolic arterial pressure measurements and heart rate determinations were made twice daily over the experimental period.

The antihypertensive activity of polythiazide was also evaluated in renal hypertensive rats prepared by the method described by Grollman(15) and treated with oral doses of 50, 100, 200 and 400 μg/kg, twice a day over a 3 or 3½ day period. Eight rats were used at each dose level. One group served as the solvent control. The drug solutions were prepared fresh daily. Blood pressure measurements in these animals were made without anesthesia by the oximetric technic(16) each morning prior to and 4 hours after drug administration.

Results. Diuretic and saluretic effects in rats. In rats polythiazide by oral administration was 2.9, 9.9, 5.5 and 0.4 times more potent than trichlormethiazide on the basis of the increase in urine volumes, Na^+, Cl^-, K^+ excretion, respectively (Fig. 1). The 95%

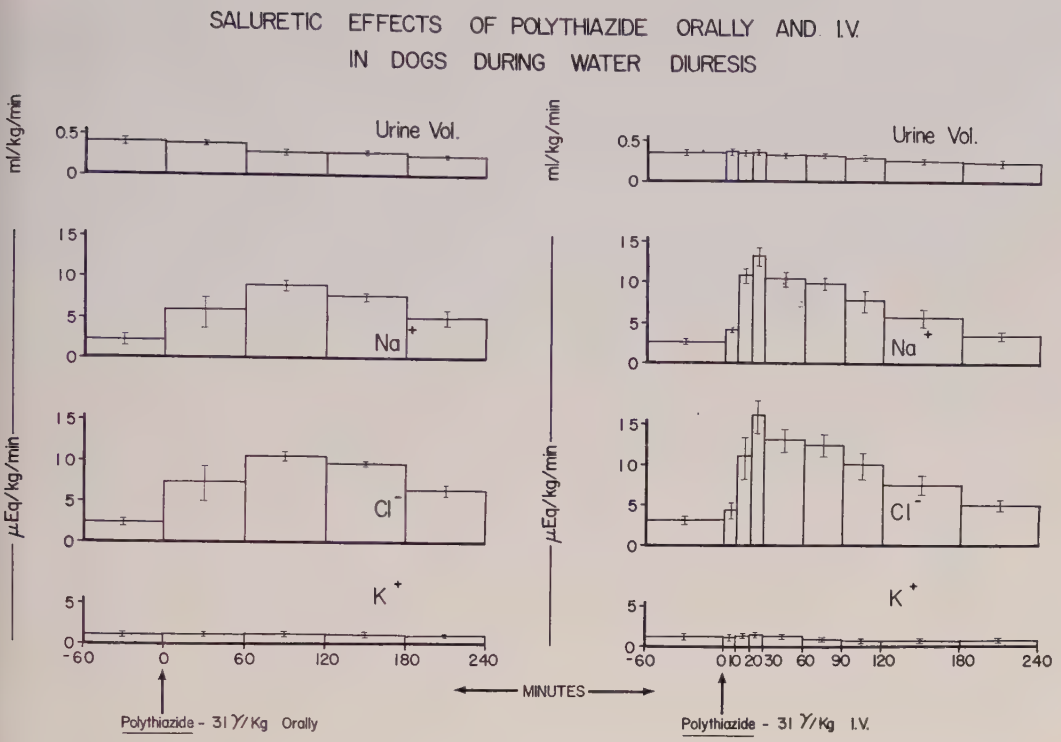


FIG. 2. Saluretic effects of polythiazide 31 μg/kg by oral and intrav. administration to trained dogs during water diuresis. Avg values for 4 dogs. Brackets indicate stand. errors.

confidence limits of the relative potency indicated the statistical significance of the differences in potency. On the basis of K^+ excretion polythiazide was less potent than trichlormethiazide whereas on the basis of Na^+ excretion it was considerably more potent.

Comparison of dose-response regression lines for polythiazide with those for benzthiazide and chlorothiazide(11) revealed that polythiazide is about 40.0 times as potent as benzthiazide or 400-600 times as potent as chlorothiazide in its natriuretic and chloruretic effects.

Saluretic effects in dogs during water diuresis. Under the conditions of water diuresis urine volumes and K^+ excretion are usually not appreciably influenced by benzthiadiazines and the effectiveness of these drugs can be evaluated only on the basis of increase in excretion of sodium and chloride. Polythiazide produced a pronounced increase in excretion of both. This increase became apparent in the first hour after oral and 20 minutes after intravenous administration of the drug (Fig. 2). In control experiments there was no increase in excretion of electrolytes during the same period.

Duration of action studies. The duration of diuretic and saluretic effects of polythiazide and trichlormethiazide is compared in Fig. 3. The difference in duration of action of polythiazide and trichlormethiazide (each at 125 μg/kg orally) became apparent in 6-24 hours after oral administration of drugs. During that period trichlormethiazide produced a rebound phenomenon—*i.e.*, a decrease in urinary excretion of Na^+ and Cl^- when polythiazide effect was still evident. The long duration of polythiazide action was observed at 31 μg/kg dose level as well as at 125 μg/kg.

Renal clearance experiments. a. *During infusion of 5% dextrose solution.* Polythiazide is an effective natriuretic agent under conditions of these experiments (Table I). Intravenous injection of 50 μg/kg followed by infusion at the rate of 75 μg/kg/hr produced a 6-8 fold increase in rate of Na^+ and Cl^- excretion. This increase was much greater than the increase in urine volumes so

TABLE I. Effect of Polythiazide i.v. on Renal Function and Electrolyte Excretion in Dogs during Infusion of Isotonic Dextrose Solution.

Avg values for 4 female dogs
Avg body wt, 17.2 kg
Anesthesia: Sodium pentobarbital, 25 mg/kg i.v.

Period	Duration, min.	Urine flow, ml/min.	Glomerular filtration rate, ml/min.	Renal plasma flow, ml/min.	Plasma conc., meq/l				pH		Rate of excretion, μeq/min.				
					Na^+	K^+	Cl^-	HCO_3^-	Urine	Plasma	Na^+	K^+	Cl^-	HCO_3^-	NH_4^+
1	20	4.1	54	144	132	2.8	98	16	6.05	7.34	42	20	38	4	17
2	20	4.0	51	135	131	2.5	99	15	5.97	7.35	43	19	43	5	19
Polythiazide, 50 γ/kg i.v. followed by 75 γ/kg/hr infusion															
3	20	4.5	51	135	128	2.4	96	16	6.01	7.34	225	36	256	6	18
4	20	4.7	50	124	131	2.3	96	15	5.89	7.35	280	38	289	4	19

that concentrations of Na^+ and Cl^- in the urine increased greatly. Excretion of K^+ was also increased. The increase was less than 1/10 of that of sodium excretion. There was no effect on excretion of bicarbonate, ammonium or on urinary pH. Polythiazide had no effect on glomerular filtration rate (creatinine clearance). Slight secondary decrease in renal plasma flow (clearance of sodium p-aminohippurate) was observed during the second clearance period after treatment. It is of questionable significance and may not be related to drug action.

b. *During infusion of isotonic NaCl solution.* Table II summarizes 4 experiments in which 10 μg/kg of polythiazide was given intravenously followed by infusion at 15 μg/kg/hr. In these experiments the rate of Na^+ excretion prior to the drug was relatively high (400-500 μEq/min) and the saluretic effects of polythiazide were less pronounced. The average increase in rate of Na^+ excretion was 143 μEq/min; that of chloride, 153 μEq/min. There was only a slight and questionable increase in urinary pH and in rates of bicarbonate, potassium and ammonium excretion. In these experiments polythiazide produced a slight increase in glomerular filtration rate and renal plasma flow. Plasma electrolytes and plasma pH were not affected by the drug. At dose levels as high as 10 mg/kg i.v. the effects of polythiazide under

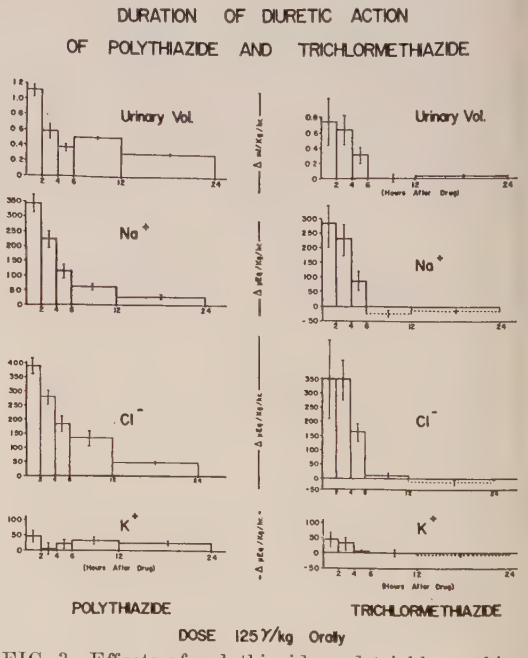


FIG. 3. Effects of polythiazide and trichlormethiazide each at 125 μg/kg orally on urinary volumes, Na^+, K^+ and Cl^- excretion over a 24-hr period in dogs deprived of food and water. Effects are expressed as differences in excretion after drug and control values for the same dogs. Avg values for 6 animals. Vertical lines represent stand. errors. Note long duration of polythiazide action.

TABLE II. Effect of Polythiazide i.v. on Renal Function and Electrolyte Excretion in Dogs during Infusion of Isotonic NaCl Solution.

Avg values for 5 female dogs
Avg body wt, 16.2 kg
Anesthesia: Sodium pentobarbital, 25 mg/kg i.v.

Period	Duration, min.	Urine flow, ml/min.	Glomerular filtration rate, ml/min.	Renal plasma flow, ml/min.	Plasma conc., meq/l				pH		Rate of excretion, μeq/min.				
					Na^+	K^+	Cl^-	HCO_3^-	Urine	Plasma	Na^+	K^+	Cl^-	HCO_3^-	NH_4^+
Avg of 1–2	20	2.7	52	188	142	3.4	108	20	7.12	7.33	486	60	466	51	8
Polythiazide, 10 γ/kg i.v. followed by 25 γ/kg/hr infusion															
3	20	3.3	60	212	141	3.4	102	—	7.26	7.31	629	73	619	—	9
4	20	2.9	55	205	146	3.2	101	19	7.20	7.34	580	61	550	60	10

same experimental conditions were not greatly different (Table III). At 10 mg/kg the natriuretic and chloruretic effects of polythiazide were not considerably greater than at lower dose levels. Even at such high doses, the effects of polythiazide on rates of potassium and bicarbonate excretion were not pronounced. The glomerular filtration rate in these experiments was not depressed even by massive doses of polythiazide.

Experimental metabolic acidosis and alkalosis. In 3 dogs with experimental acidosis, 0.4 mg/kg of polythiazide followed by infusion at 0.6 mg/kg/hr produced a pronounced increase in excretion rate of sodium and chloride, slight increase in excretion of potassium, and no effect on rate of ammonium excretion. There was a slight and questionable decrease in urinary pH and in rate of bicarbonate excretion. A typical experiment on an acidotic dog is presented in Table IV.

In dogs with experimental alkalosis, polythiazide at 0.4 mg/kg followed by infusion at 0.6 mg/kg/hr produced a pronounced increase in rate of Na^+ and Cl^- excretion. There was a slight increase in excretion rate of K^+. Under conditions of metabolic alkalosis polythiazide produced a considerable increase in rate of bicarbonate excretion (Table V).

Carbonic anhydrase inhibitory activity in vitro. The *in vitro* molar concentration of polythiazide required for 50% inhibition of carbonic anhydrase was estimated as 5×10^{-7}M and that for chlorothiazide 1×10^{-6}M.

Cardiovascular effects of polythiazide. Anesthetized normotensive dogs. In normotensive anesthetized dogs, polythiazide was tested at dose levels of 1 to 50 mg/kg, intravenously. The animals were observed for at least 4 hours following drug administration. There were no significant changes in blood pressure or heart rate which could be attributed to the drug action. Slight potentiation of the pressor response of adrenaline was noted occasionally.

Unanesthetized hypertensive dogs. In 4 dogs with experimental or spontaneous hypertension, daily oral administration of polythiazide (400 μg/kg/day for 5 days) caused decrease in both systolic and diastolic pressure, the maximum effect seemed to appear on the fifth day of treatment (Fig. 4), when the decrease in systolic and diastolic pressure was significantly ($P<0.05$) lower in the treated than in the control group of 4 other hypertensive dogs. Heart rate recorded concomitantly with arterial pressure deter-

TABLE III. Effect of Polythiazide on Glomerular Filtration Rate and Electrolyte Excretion during Infusion of Isotonic NaCl Solution.

Dog ♀, 11.4 kg
Anesthesia: Sodium pentobarbital, 25 mg/kg i.v.

Period	Duration, min.	Urine flow, ml/min.	Glomerular filtration rate, ml/min.	Plasma conc., meq/l				pH		Rate of excretion, μeq/min.			
				Na^+	K^+	Cl^-	HCO_3^-	Urine	Plasma	Na^+	K^+	Cl^-	HCO_3^-
1	20	1.0	28	145	3.9	120	25	7.12	7.17	149	21	158	29
2	20	.9	28	151	3.8	118	23	7.19	7.27	145	20	146	27
			Polythiazide, .1 mg/kg prime followed by .1 mg/kg/hr infusion										
3	20	1.8	—	150	3.9	120	—	7.20	7.26	340	50	305	—
4	20	1.4	33	151	3.7	120	22	7.20	7.24	217	32	228	39
			Polythiazide, 1 mg/kg prime followed by 1 mg/kg/hr infusion										
5	20	1.8	—	145	4.0	120	—	7.30	7.27	331	48	305	—
6	20	1.6	33	151	3.7	122	21	7.20	7.28	390	39	267	51
			Polythiazide, 10 mg/kg prime followed by 10 mg/kg/hr infusion										
7	20	2.4	—	146	3.5	120	—	7.30	7.25	463	62	407	—
8	20	2.0	34	152	3.8	120	20	7.40	7.25	374	56	345	75

minations was not significantly changed by the polythiazide treatment.

Unanesthetized hypertensive rats. Daily oral administration of 100 μg/kg, or more, of polythiazide produced a statistically significant ($P<0.05$) lowering of mean arterial blood pressure in hypertensive rats (Fig. 5). The antihypertensive effect did not become apparent until the second or third days of treatment.

Discussion. Our studies in rats and dogs on the pharmacological activities of polythiazide indicated that this compound is a potent, orally active diuretic and antihypertensive agent. The long duration of action, increase in excretion of sodium and chloride in equivalent amounts, effectiveness in metabolic acidosis and alkalosis characterized the diuretic activity of polythiazide. Almost equal activity by oral and intravenous administration indicated complete absorption. The potency of polythiazide was estimated on the basis of a 5 hour urine collection in rats. Because of the prolonged duration of polythiazide action its actual potency is probably considerably higher than these data indicated. But even on the basis of a 5 hour observation period polythiazide is one of the most potent diuretics known. The exceptionally long duration of polythiazide action shown in this study was confirmed in recent clinical investigation(17). Polythiazide was found to be effective in man for as long as 48 hours after oral administration.

Polythiazide produced a pronounced increase in sodium and chloride excretion in dogs infused with a 5% dextrose solution. Glomerular filtration rate in these experi-

TABLE IV. Effect of Polythiazide i.v. on Excretion of Electrolytes in a Dog with Experimental Metabolic Acidosis.

Dog ♀, 11.5 kg
Anesthesia: Sodium pentobarbital, 25 mg/kg i.v.

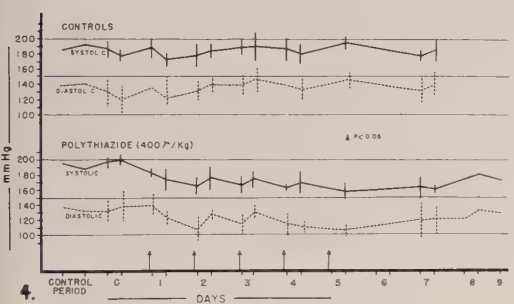
Period	Duration, min.	Urine flow, ml/min.	Glomerular filtration rate, ml/min.	Plasma conc., meq/l				pH		Rate of excretion μeq/min.				
				Na^+	K^+	Cl^-	HCO_3^-	Urine	Plasma	Na^+	K^+	Cl^-	HCO_3^-	NH_4^+
Avg of 1–2	20	2.1	30	145	2.8	108	11	5.3	7.15	36	12	92	.4	38
			Polythiazide, 0.4 mg/kg i.v. followed by infusion at 0.6 mg/kg/hr											
3	20	2.1	29	143	2.6	105	13	5.1	7.13	57	14	164	.2	36
4	20	1.9	30	149	2.6	95	12	5.0	7.13	158	24	222	.2	38
5	20	1.5	27	141	2.6	103	13	4.8	7.18	123	21	178	.1	37

TABLE V. Effect of Polythiazide i.v. on Excretion of Electrolytes in a Dog with Experimental Metabolic Alkalosis.

Dog No. 6086 ♀, 22.2 kg
Anesthesia: Sodium pentobarbital, 25 mg/kg i.v.

Period	Duration, min.	Urine flow, ml/min.	Glomerular filtration rate, ml/min.	Plasma conc., meq/l				pH		Rate of excretion, μeq/min.			
				Na^+	K^+	Cl^-	HCO_3^-	Urine	Plasma	Na^+	K^+	Cl^-	HCO_3^-
1	20	4.5	60	148	2.6	99	28	7.7	7.46	88	35	34	98
2	20	4.2	56	147	2.6	97	28	7.7	7.48	90	31	40	93
Polythiazide, 0.4 mg/kg followed by 0.6 mg/kg/hr infusion													
3	20	5.4	60	150	2.7	97	31	7.8	7.45	438	44	237	214
4	20	5.8	59	149	2.7	99	30	7.8	7.47	465	48	252	243
5	20	6.3	61	153	2.7	96	32	7.8	7.47	504	53	265	286

ments was not increased. The inhibition of carbonic anhydrase which is observed *in vitro*, was not generally evident during the polythiazide diuresis seen in normal animals, even at doses as high as 10 mg/kg i.v. Only in experimental alkalosis was there a marked increase in bicarbonate excretion after polythiazide, attributable to the carbonic anhydrase inhibitory activity of the drug.



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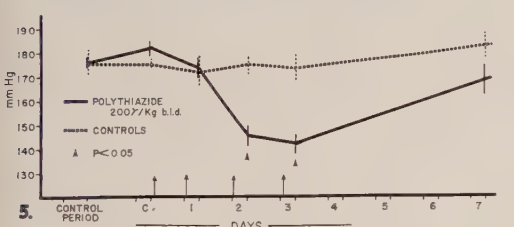

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FIG. 4. Effect of polythiazide, 400 μg/kg orally, on blood pressure and heart rate of unanesthetized, hypertensive dogs. At the arrow polythiazide was administered; C is control. Avg values for 4 dogs. Vertical bars represent stand. errors of mean. Where indicated (▲) there is a statistically significant difference between control and treated animals.

FIG. 5. Effect of oral treatment with 200 μg/kg of polythiazide twice daily on blood pressure in unanesthetized hypertensive rats. At the arrow polythiazide was administered; C is control. Avg for 8 rats. Vertical bars represent stand. errors of mean. Where indicated (▲) there is a statistically significant difference between control and treated rats.

The demonstration of antihypertensive activity of polythiazide in animals indicated the possible usefulness of this drug in treatment of hypertension. The sustained effect should be a desirable property of polythiazide used as an antihypertensive agent. The mechanism of antihypertensive action of benzthiadiazines cannot yet be clearly defined. The reduction of blood volume or decrease in reactivity of blood vessels as a result of blood volume reduction(18) are still favored explanations for the mechanism of the benzthiadiazines action in hypertension. The changes in intracellular concentrations of Na^+ or K^+ in the arterial walls may, on the other hand, also explain the antihypertensive activity(19). It was recently postulated(20), that ischemic kidneys of rats may produce a substance which would tend to increase selectively the intracellular K^+ concentration in the arterial walls of hypertensive rats. It is conceivable that a drug like polythiazide may antagonize the effect of this hypothetical substance on redistribution of tissue electrolytes.

Summary. Polythiazide was found to be a highly potent, orally active diuretic agent in rats and dogs. Duration of polythiazide action was prolonged. The diuretic effect was demonstrated as late as 12 to 24 hours after oral administration of the drug. Onset of polythiazide action was rapid. After intravenous administration to dogs, the maximal saluretic effect was obtained in 10 to 20 minutes. The onset of action after oral administration was less than one hour; maximal ef-

fect was obtained either during the first or second hour. Polythiazide produced an almost equal increase in excretion of sodium and chloride. The increase in excretion of potassium was approximately 1/10 of that of sodium. In acute experiments on rats polythiazide was found to be 10 times more potent than trichlormethiazide in its natriuretic effect but only 0.4 times as potent in its kaliuretic effect. Polythiazide at dose levels as high as 10 mg/kg i.v. did not depress glomerular filtration rate. Polythiazide is a carbonic anhydrase inhibitor *in vitro*. *In vivo*, polythiazide produced no or only minimal increases in excretion of bicarbonate in animals with normal acid-base balance. The drug was effective as saluretic agent in dogs with experimental metabolic acidosis or alkalosis. Antihypertensive activity of polythiazide was clearly demonstrated in rats and dogs with experimental hypertension. The antihypertensive effect had a slow onset and became evident on the second day of treatment.

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Demonstration of Countercurrent Diffusion Exchange in the Vasa Recta of the Renal Medulla.* (26781)

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Man and certain animals are capable of producing urine hypertonic to plasma as a means of conserving body water. It is currently held that, under the influence of antidiuretic hormone, the collecting ducts become more permeable to water and probably to urea. Urine passing through the collecting duct equilibrates with the hypertonic interstitium of the renal medulla and papilla, becoming concentrated with respect to plasma (1). Not only is the medulla hypertonic, but a gradient of osmolality(2) and solute concentration (sodium, urea, chloride, creatinine) has been demonstrated, increasing from the base of the medulla to the papillary tip(1,3,4). Micropuncture studies have

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shown that fluid in the tip of the Henle loop, plasma in the vasa recta, and urine in the collecting ducts are all hypertonic to plasma and are of approximately the same osmolality at any one level in the concentrating kidney(5,6). This gradient is presently considered maintained by an active sodium pump, primarily in the loop of Henle, with countercurrent diffusion exchange of sodium, urea, water and other diffusible solutes in the vasa recta(1,6). Some sodium also probably enters the medulla from the collecting ducts (7).

The close approximation of the descending and ascending limbs of the vasa recta in the outer medulla, with blood flowing in opposite directions, could allow countercurrent exchange of diffusible substances to which the vessels are permeable(1,3). This exchange would serve to decrease the effective flow of interstitial diffusible solute out of the papilla, by shunting it from the ascending to descending limbs of the vasa recta. At the same time water would be partially excluded from the papilla by exchange from the descending to ascending limbs. This would serve to maintain the high solute concentrations in the papilla. Since the process is passive, diffusible solute present in the renal arterial blood should be short circuited from descending to ascending limbs of the vascular bundles. This would effectively decrease their incorporation rate into the deeper papillary areas.

The following experiment was devised to determine whether a rapidly diffusible ion to which capillaries are permeable *viz.* Rb^{86+}, is in fact incorporated more slowly into the renal papilla than a relatively nondiffusible substance, I^{131} human serum albumin.

Method. Six hydropenic mongrel dogs each weighing approximately 10 kg were anesthetized with about 25 mg/kg of thiopental sodium or Na-ethyl-(1-methyl-propyl)-malonyl-thio-urea (Inactin).‡ The right carotid artery was exposed, a polyethylene catheter inserted into the vessel, and the tip advanced to the root of the aorta. One femoral artery was catheterized for collection of blood samples. Through an abdominal incision both renal pedicles were isolated and a loose ligature placed about each. Each ureter was isolated and a small polyethylene catheter threaded to the renal pelvis, from approximately 6 cm below the pedicle. After allowing time for stabilization of the animal, a mixture of I^{131} albumin and $Rb^{86}Cl$ in normal saline, containing a small amount of nonradioactive rubidium, was infused into the aortic root. A constant infusion pump delivered approximately 15 μc/sec of $Rb^{86}Cl$ and 10 μc/sec of I^{131} albumin. Twenty seconds after beginning the infusion, the renal pedicles were simultaneously ligated. The kidneys were then removed, and immediately placed in a dry ice-acetone mixture. Continuous femoral arterial blood samples were collected each second for 25 seconds during infusion. Duplicate pieces of inner medulla (papilla), outer medulla (carefully excluding the cortico-medullary border zone) and cortex, were cut from the frozen kidneys and weighed. Rb^{86+} and I^{131} activity in these pieces, as well as that in aliquots of arterial plasma was determined, employing a well-type scintillation counter and gamma ray spectrometer. Tissue radioactivities for Rb^{86+} and I^{131} were expressed as counts per gram of tissue (wet weight), and plasma radioactivity as counts per milliliter of plasma. After correcting for collecting catheter time delay, mean arterial blood plasma Rb^{86+} and I^{131} radioactivity concentrations were determined by summing the plasma concentrations from zero time until time of ligation and dividing by this time interval. The ratio of tissue radioactivity to average arterial plasma radioactivity for Rb^{86+} and I^{131} was individually calculated, multiplied by 100 and designated "V", "apparent volume of distribution," in ml/100 g of tissue. Ratios of the apparent volume of distribution of Rb^{86+} (V_R) to that of I^{131} (V_I) were determined for each piece of tissue (V_R/V_I). Timed urine specimens were collected from each ureter for osmolality and volume flow determinations. Urine to plasma osmolal ratios varied from 2.0 to 3.6. Arterial pressure was monitored by means of a strain gauge transducer and direct writing recorder throughout the procedure. Mean pres-

‡ Promonta GmbH, Hamburg, Germany.

TABLE I. Ratio of "Apparent Volume of Distribution" of $Rb^{86}Cl$ and I^{131} Albumin (V_R/V_I) in the Kidney in 20 Seconds.

Kidney No.	Inner medulla (papilla)	Outer medulla	Cortex
1 R	.197	.700	3.19
L	.216	1.03	3.59
2 R	.789	2.40	7.33
L	.566	1.55	5.64
3 R	.484	3.03	6.68
L	.441	2.46	7.18
4 R	.725	1.05	3.29
L	.641	.737	3.16
5 R	.322	1.93	6.49
L	.239	1.5	2.9
6 R	.64	2.6	7.2
L		1.8	4.9
Mean ± S.D.	.48 ±.20	1.73 ±.55	5.13 ±1.74

sure remained above 105 mm/Hg in all animals.

Three additional hydropenic dogs were similarly prepared but $Na^{22}Cl$ was used in place of $Rb^{86}Cl$ and the kidneys were ligated in 15 seconds. Only cortex and papilla were analyzed in these animals.

Results. The ratios of Rb^{86+} to albumin volumes of distribution in 20 seconds (V_R/V_I) averaged 0.48 ± 0.20 for the papilla, 1.73 ± 0.55 for the outer medulla and 5.13 ± 1.74 for the cortex (Table I). It is apparent that less than one half as much rubidium as albumin was incorporated into the papilla in 20 seconds, while approximately twice as much rubidium appeared in the outer medulla, and 5 times as much in the cortex. Similarly, the Na^{22+} to albumin volumes of distribution in 15 seconds (V_{Na}/V_I) averaged 0.64 ± 0.16 for the papilla and 2.05 ± 0.45 for the cortex.

Discussion. The ascending and descending limbs of the vasa recta, comprising the vascular bundles of the outer medulla, could act either as conduits carrying blood to and from the papilla, or as permeable vessels acting as countercurrent diffusion exchangers. If they were merely conduits, then no Rb^{86+} could escape into the interstitium of the outer medulla and the ratio V_R/V_I in the outer medullary tissue could only be 1.0. The papillary tissue in this instance would also have a V_R/V_I ratio of 1.0, for no Rb^{86+} could escape prior to reaching this area. Furthermore, none could accumulate in excess of albumin, since the 20 second perfusion time is substantially less than the papillary circulation time (8). However, as the results indicate, the observed ratio of V_R/V_I in the papilla was always less than 1.0. This circumstance can only mean that some Rb^{86+} has escaped from the descending vessels, prior to entering the papilla. That the descending and ascending limbs of the vasa recta are permeable to Rb^{86+} is further substantiated by the average V_R/V_I ratio of 1.75 found in the outer medulla. Such a situation suggests diffusion of Rb^{86+} into the interstitium, while the albumin remains intravascular. In 2 kidneys, 1R and 4R (Table I) the observed outer medullary V_R/V_I ratios were 0.70 and 0.74, *i.e.*, there was less Rb^{86+} than albumin in these pieces. The segment removed from the medulla in these cases was probably far enough distal to the origin of the vascular bundles at the base, to have allowed some diffusion loss of Rb^{86+} from the blood prior to its reaching the tissue counted.

The accumulations of Rb^{86+} and I^{131} in the cortex cannot be interpreted in the same manner as those in the papilla because of the very rapid blood flow in this area(8). The large V_R/V_I ratios do, however, indicate permeability of the cortical capillaries to the Rb^{86} ion.

In the 3 dogs in which Na^{22+} was used, the findings are similar to that found with Rb^{86+} and indicate that the Na^{22} ion was also effectively excluded from the papilla.

These results, plus the anatomic arrangement of the medullary vessels, demonstrate that countercurrent exchange can occur in

TABLE II. Ratio of "Apparent Volume of Distribution of $Na^{22}Cl$ and I^{131} Albumin (V_{Na}/V_I) in the Kidney in 15 Seconds.

Kidney No.	Inner medulla (papilla)	Cortex
7 R	.82	1.80
L	.77	2.14
8 R	.43	1.64
L	.67	2.80
9 R	.54	1.9
Mean ± S.D.	.64 ± .16	2.05 ± .45

the vascular bundles. There is a marked similarity, as also demonstrated by the electron microscope, between vascular structure and arrangement in the medulla to that in the swim bladder of certain deep sea fish(9, 10). In the latter, countercurrent diffusion exchange of gases, primarily oxygen does take place(11).

The findings of solute and osmolal gradients in the papilla, and especially the high concentrations in the vasa recta, as shown by the micropuncture studies of Wirz(5) and confirmed by Gottschalk(6) have been explained by countercurrent diffusion exchange in these vessels. Recent micropuncture studies by Thurau, Sugiura, and Lilienfield(12) have demonstrated albumin concentrations in the vasa recta at the tip of papillary loop nearly 3 times greater than those in the straight vessels at the base of the papilla. These results are interpreted to indicate a proximal water shunt by countercurrent diffusion. White, Tosteson and Rolf(13) showed that THO was incorporated more slowly into the inner medulla than Na^{22+}, again indicating a proximal water shunt in the vascular bundle. Lassen and Longley working with rats have shown that Kr^{85}, Na^{24+} and I^{131} iodoantipyrine were effectively excluded from the papilla as compared to the cortex(14). Furthermore, the efficiency of exclusion seemed to be proportional to the expected diffusibilities of these substances, *i.e.*, the more rapidly diffusible were more effectively excluded from the papilla.

Summary. The accumulations of Rb^{86+} and I^{131} albumin were compared in the cortex, outer medulla and papilla of 6 hydropenic anesthetized mongrel dogs. Only one-half as much rubidium as albumin appeared in the papilla in 20 seconds of perfusion, while almost twice as much appeared in the outer medulla and 5 times as much in the cortex. In 3 hydropenic dogs perfused with $Na^{22}Cl$ and I^{131} albumin the Na^{22+} was similarly excluded from the papilla. These data suggest that the vasa recta do indeed function as countercurrent exchangers to maintain the high renal medullary solute concentrations and gradient, necessary to conserve body water.

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Iron Replacement of Lactalysate and Embryo Extract in Growth of Cell Cultures.* (26782)

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A serumless medium for the growth of mammalian cell cultures has been described (1). A critical constituent and the only undefined component of this medium was lactalysate, a commercial enzymic digest of lactalbumin. In further studies, requirements of avian cell cultures for serum and embryo extract were partially replaced by components of the serumless medium. A specific function performed by lactalysate and by embryo extract was the counteraction of a toxicity of serum for the avian cells(2).

The present communication describes the effectiveness of iron and iron-bearing materials in replacement of lactalysate and embryo extract requirements for growth of cell cultures and for overcoming a serum toxicity.

Materials and methods. Procedures have been described previously(1,2,3). In the present studies, 5000 Walker carcinosarcoma 256 cells from stock cultures or 60 to 80,000 white Leghorn chick embryo (14 day) lung cells prepared by trypsinization were established (plated) overnight in 1 ml of medium in 15 × 125 mm roller tubes placed in an inclined position in racks. Incubation was at 37°C in an atmosphere of 92% air—8% carbon dioxide. After 16 to 24 hours, the medium was withdrawn from the tubes which were then rinsed with Earle's solution. One ml of test basal medium containing graduated quantities of test substances at 6 to 9 levels was pipetted into duplicate tubes. Repeated experiments confirmed the results in all cases. Incubation was continued for 3 or 4 days. The cultures were examined microscopically and growth was measured by protein determination according to the procedure of Oyama and Eagle(4).

The medium used to establish Walker tumor cells contained 0.2 mM pyruvate, 10 μg adenosine per ml, 5% bovine serum and amino acids, vitamins, glucose, minerals, antibiotics and phenol red in the quantities specified for serumless medium(1). This medium, supplemented with 5% embryo extract, was used to establish chick embryo cultures.

The test basal medium for Walker carcinosarcoma 256 cells was the serumless medium described previously(1) with omission of mucic acid and lactalysate and with replacement of Tween 80 by Tween 20. The latter material has been found in this laboratory to be less toxic than Tween 80 for cell cultures (5). Methyl oleate was decreased to 10 μg/ml. This test basal medium, modified to contain 5 μg methyl oleate and 5 μg insulin per ml, was utilized for studies of avian cultures.

Transferrin† was prepared by modification of the procedure of Surgenor *et al.*(6) from pooled human plasma. Bovine hemoglobin (2 × recrystallized) and horse heart cytochrome C (type III) were obtained from Sigma Chemical Co., St. Louis, Mo. Horse heart myoglobin and horse spleen ferritin were obtained from Pentex Incorporated, Kankakee, Ill.

Lactalysate was Edamin DR, an enzymatic digest of lactalbumin supplied by the Sheffield Chemical Co., Norwich, Conn.

The relatively non-dialyzable portion of lactalysate (lactalysate residue) was prepared by dialysis overnight against running tap water followed by dialysis for 24 hours against several changes of an excess of demineralized water. The lactalysate residue was freeze-dried. Hydrolysates of lactalysate residue were prepared by autoclaving 100 mg in 5 ml 6 N HCl in sealed tubes for 16 hours at 15 lb pressure. The HCl was removed by flash evaporation. The hydroly-

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† Kindly supplied by Dr. B. F. Sanders, Merck, Sharp and Dohme, West Point, Pa.

TABLE I. Growth Response of Walker Carcinosarcoma 256 Cells to Lactalysate, Lactalysate Residue, Hydrolyzed Residue, Ash and Ferric Nitrate.

Lactalysate		Lactalysate residue		Hydrolyzed lactalysate residue		Lactalysate ash		Lactalysate residue ash		Fe^{+++}	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml*	μg cell protein/tube	μg/ml†	μg cell protein/tube	μg/ml	μg cell protein/tube
0	4	0	4	0	4	0	4	0	4	0	4
100	6	5	26	5	32	600	6	50	4	.056‡	42
200	44	10	46	10	40	1,200	8	100	5	.28	51
500	73	20	69	50	64	3,000	13	200	6	.56	57
1,000	74	50	52	50	59	6,000	36	500	11	1.12	59
2,000	66	100	59	100	57	15,000	12	1,250	19	2.24	47
										4.48	14

Inoculum was 5000 cells/ml. Growth period was 72 hr.

* Equivalent of lactalysate. † Equivalent of lactalysate residue. ‡ Equivalent to a concentration of 0.001 mM ferric nitrate.

Individual data points represent means of 2 tubes. Avg stand. dev. per single point was estimated to be ± 5.2 μg cell protein/tube. Avg stand. error of individual means was estimated to be ± 3.7 μg cell protein/tube.

sate was taken up in ethanol and again flash evaporated. An aqueous solution of the hydrolysate then was decolorized with Darco charcoal, grade S-51.

Iron determination was by the procedure of Gubler *et al.*(7).

Results. The comparative stimulatory effects on growth of Walker carcinosarcoma 256 cells by lactalysate, lactalysate residue, lactalysate residue hydrolysate, ash preparations and ferric nitrate are shown in Table I. It is apparent that the growth-promoting effect of lactalysate was concentrated in the non-dialyzable residue which comprised about 8% of the original material. The dialysate was inactive. The activity of the lactalysate residue persisted after drastic hydrolysis. The ash of both lactalysate and lactalysate residue contained slight activity, but excessive inactive salts present might have interfered with the bioassays. A number of trace elements were tested for activity, and iron as ferric nitrate was found to stimulate growth markedly. The iron content of lactalysate and lactalysate residue were determined to be 0.011% and 0.091% respectively, which was sufficient to account for activity on the basis of iron content. Salts of manganese, zinc, copper, and cobalt, as well as sodium nitrate, were inactive either alone or in combination over a wide range of concentrations.

The growth response of Walker carcinosarcoma 256 to lactalysate, transferrin, and several iron-containing proteins and compounds is shown in Table II. Minute quantities of transferrin, hemoglobin, myoglobin and hemin stimulated growth. Inhibition was produced at higher levels. Substantially greater concentrations of cytochrome C were required for stimulation. In further investigation, sodium iron versenate stimulated at 1-5 μg/ml. Ferrocyanide, ferricyanide, catalase, and ferritin were inactive.

Lactalysate, lactalysate residue, lactalysate residue hydrolysate and embryo extract earlier were observed to stimulate chick embryo cell cultures as well as to overcome a toxicity of bovine or chicken serum(2). Continuation of the present studies demonstrated that iron and iron-bearing materials substi-

TABLE II. Growth Response of Walker Carcinosarcoma 256 Cells to Various Iron-Bearing Materials.

Lactalysate		Human transferrin		Bovine hemoglobin		Horse myoglobin		Horse cytochrome C		Hemin	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube
0	10	0	10	0	10	0	10	0	10	0	10
100	30	.1	18	1	15	1	22	1	11	.1	11
200	45	.5	32	2	22	2	29	2	8	.5	36
500	51	1	43	5	36	5	46	5	13	1	22
1000	50	5	47	10	45	10	52	10	13	2	6
2000	32	10	46	50	31	50	54	50	15	5	4
		50	41	100	32	100	6	100	13	10	6
				500	6	500	6	500	46		
								1000	39		

Inoculum was 5000 cells/ml. Growth period was 72 hr.

Individual data points represent means of 1 to 3 tubes. Avg stand. dev. per single point was estimated to be ± 5.3 μg cell protein/tube. Avg stand. error of individual means estimated to be ± 3.7 μg cell protein/tube.

tuted for lactalysate and derivatives and for embryo extract in promotion of growth and inhibition of the toxic serum effect (Table III). Iron, hemoglobin, and myoglobin supported growth comparable to that with lactalysate residue (superior to lactalysate for avian cultures) or with 5% dialyzed embryo extract. Of particular interest was the ineffectiveness of human transferrin. As with the Walker tumor cells, 0.5 to 1 μg hemin, 1-5 μg sodium iron versenate and 500-1000 μg cytochrome C stimulated, while ferrocyanide, ferricyanide, catalase, and ferritin were inactive. These effects were most notable in media containing bovine serum. Additional toxicity perhaps of a different nature in chick serum was observable even after addition of embryo extract(3) and diminished responses to iron-bearing materials in the presence of this serum.

In Fig. 1 is shown the appearance of the chick embryo lung cultures in different media. Fig. 1a is the plated inoculum. Growth in basal serumless medium (Fig. 1b) is improved by either lactalysate residue (Fig. 1c) or by ferric nitrate (Fig. 1d). Inclusion of 5% dialyzed bovine serum in the basal medium results in destruction of the cells (Fig.

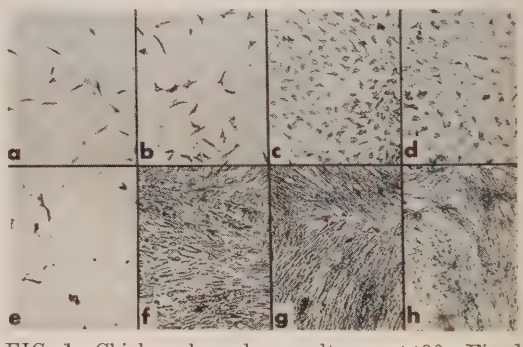

FIG. 1. Chick embryo lung cultures. ×30. Fixed with Bouin's and stained with Giemsa. Inoculum was 80,000 cells/ml. Growth period was 4 days.

(a) Inoculum plated 24 hr.
(b) Basal medium devoid of lactalysate or iron.
(c) Basal medium + 250 μg lactalysate residue/ml.
(d) Basal medium + ferric nitrate at 0.02 mM.
(e) Basal medium + 5% dialyzed bovine serum.
(f) Basal medium + 5% dialyzed bovine serum & 5% dialyzed embryo extract.
(g) Basal medium + 5% dialyzed bovine serum & 250 μg lactalysate residue/ml.
(h) Basal medium + 5% dialyzed bovine serum & ferric nitrate at 0.02 mM concentration.

TABLE III. Growth Response of Chick Embryo Lung Cells to Various Iron-Bearing Materials.

Lactalysate residue		Fe^{+++}		Human transferrin		Bovine hemoglobin		Horse myoglobin	
μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube	μg/ml	μg cell protein/tube
Basal medium without added dialyzed bovine serum									
0	19	0	19	0	19	0	19	0	19
10	58	.028*	34	1	20	2	46	2	54
50	72	.056	46	5	17	10	72	10	68
100	78	.28	64	10	12	20	72	20	74
250	78	.56	80	50	14	50	82	50	82
500	86	1.12	74	100	12	100	94	100	86
1000	90	2.8	80	500	16	500	108	500	94
Basal medium with 5% dialyzed bovine serum									
0	30	0	30	0	30	0	30	0	30
10	94	.028*	36	1	38	2	64	2	80
50	158	.056	34	5	32	10	78	10	80
100	166	.28	94	10	30	20	124	20	134
250	184	.56	128	50	36	50	146	50	180
500	190	1.12	158	100	30	100	158	100	172
1000	170	2.8	158	500	28	500	118	500	160

Inoculum was 80,000 cells/ml. Growth period was 4 days. Growth in basal medium plus 5% dialyzed embryo extract = 76 μg cell protein/tube. Growth in basal medium plus 5% dialyzed embryo extract plus 5% dialyzed bovine serum = 188 μg cell protein/tube.

* Equivalent to concentration of 0.0005 mM ferric nitrate.

Individual data points represent means of 2 tubes. Avg stand. dev. per single point for basal media without added dialyzed bovine serum was ± 4.1 μg cell protein/tube. Avg stand. error of individual means was estimated to be ± 2.9 μg cell protein/tube. For basal medium with dialyzed bovine serum, avg deviation was estimated to be ± 12.1 μg cell protein/tube. Avg stand. error of individual means estimated to be ± 3.6 μg cell protein/tube.

1e). This is readily overcome by lactalysate residue (Fig. 1g), dialyzed chick embryo extract (Fig. 1f), or by ferric nitrate (Fig. 1h).

Discussion. The stability of a growth-promoting factor of lactalysate to drastic hydrolytic conditions and detection of activity in ashed samples, despite possible interference of excessive salts, suggested that trace metals might be stimulatory. Iron and iron-bearing compounds, indeed, were found to simulate the effects of lactalysate and derived products in 2 different culture systems. Growth of both Walker carcinosarcoma 256 cells in serumless medium and chick embryo lung in a similar medium with or without added serum was stimulated. Furthermore, like lactalysate and embryo extract, iron and iron-bearing materials specifically overcame a toxic effect of serum through which chick embryo cells failed to multiply and underwent dissolution. The retention of activity in the non-dialyzable portion of lactalysate and embryo extract indicated that these residues bind, stabilize or transport iron; in fact, following dialysis, the iron content of lactalysate residue was increased approximately 8 to 9-fold and corresponded to an increased activity. It is evident that at least one major contribution of lactalysate and dialyzed embryo extract to cell growth was related to iron requirements. Of course, embryo extract and lactalysate may provide amino acids and vitamins to cultures in media in which these nutrients are not otherwise incorporated. The growth of the Walker tumor cells with lactalysate or chick embryo lung cells with lactalysate residue or embryo extract often was slightly superior to that obtained with other supplements as may be seen from the tables. This suggests that the properties of these materials serve particularly well the processes of iron metabolism or contribute additional special nutrients or non-specific factors such as buffering capacity. Other functions that might be served by lactalysate preparations and embryo extract as well as their effects on other cell strains and cells in long term culture are at present under investigation.

In earlier work, Morgan, Morton, and Parker included iron in their Medium 199 de-

signed to prolong survival of chick embryo cultures(8). White observed beneficial effects on strain "L" mouse fibroblasts in semi-synthetic media(9). Waymouth stimulated growth of these cells in synthetic media with iron(10). Ham found iron to be essential for growth of isolated Chinese hamster cells in media containing serum albumin and fetuin(11). The current studies quantitatively confirm these findings on mammalian and avian cell cultures. Hemoglobin also has been observed earlier to stimulate growth of cell cultures(12,13,14).

A marked difference between mammalian and avian cells was the incapacity of the latter to respond to human transferrin. The toxic sera presumably already contained high concentrations of transferrin which was not utilized by the avian cells or actually was involved in the toxicity of the serum through interference with availability of minute quantities of iron.

Summary. Iron salts and iron-bearing materials replaced lactalysate in stimulation of growth of Walker carcinosarcoma 256 cells in serumless medium and replaced lactalysate and embryo extract in stimulation of chick embryo lung cultures in a similar medium with or without further addition of serum. Presumptive evidence is presented that a stimulatory activity of lactalysate is due to iron and iron-bearing materials. A specific function of lactalysate and embryo extract in overcoming toxicity of serum for embryo cell cultures was served equally well by iron or iron-bearing materials. The avian cells revealed in their incapacity to respond to transferrin a characteristic distinguishing them from the Walker tumor cells.

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Mammary Lobulo-Alveolar Growth Induced by Anterior Pituitary Hormones in Adreno-Ovariectomized and Adreno-Ovariectomized-Hypophysectomized Rats.*† (26783)

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Studies in ovariectomized-hypophysectomized or in adreno-ovariectomized-hypophysectomized rats have indicated that hormones from the anterior pituitary and ovaries are both essential for inducing mammary lobulo-alveolar growth(1-3). The adrenals are believed to be of secondary importance, although it is recognized that they secrete several steroids which can influence mammary growth(3). Only a small degree of mammary growth was produced by injecting anterior pituitary extracts into ovariectomized

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TABLE I. Induction of Mammary Lobulo-Alveolar Growth by Anterior Pituitary Hormones in Adreno-Ovariectomized and Adreno-Ovariectomized-Hypophysectomized Rats.

Group & No. of animals	Treatment* (3× daily; 10 days)	Mammary rating†					Avg body wt (g)		
		I	II	III	IV	V	Initial	Final	Difference
	Adreno-ovariectomized rats								
1 (9)	.1 ml saline	3	6	—	—	—	222	225	+ 3
2 (7)	2 mg STH	—	2	5	—	—	217	260	+43
3 (7)	30 I.U. prol.	—	—	4	3	—	208	222	+14
4 (7)	1.33 mg STH + 20 I.U. prol.	—	—	—	1	6	220	261	+41
5 (10)	20 mg A.P.	—	—	2	5	3	224	244	+20
	Adreno-ovariectomized-hypophysectomized rats								
6 (9)	.1 ml saline	6	3	—	—	—	211	212	+ 1
7 (9)	1.33 mg STH + 20 I.U. prol.	—	—	4	5	—	182	225	+43

* STH = Growth hormone; prol. = prolactin; A.P. = anterior pituitary powder.
† See text for mammary rating system.

or adreno-ovariectomized rats(4). Recently, Clifton and Furth(5) observed complete lobulo-alveolar development in adreno-gonadectomized male rats of the Fischer strain after implanting "mammotropic" pituitary tumors intramuscularly. No data were given as to completeness of adrenalectomy in these rats. Since these pituitary tumors apparently secrete both STH and prolactin(5), it was of interest to determine whether frequent injections of large doses of these 2 hormones or of whole anterior pituitary powder could induce lobulo-alveolar mammary growth in adreno-ovariectomized or adreno-ovariectomized-hypophysectomized rats.

Methods. Mature virgin female Carworth rats of the CFN strain, weighing 180-240 g each, were used in this study. Rats in Groups 1-5 (Table I) were adreno-ovariectomized (doubly operated) while rats in Groups 6 and 7 were hypophysectomized by the parapharyngeal approach and 3 days later were adreno-ovariectomized (triply operated). Following each operation, the rats were injected once subcutaneously with 30,000 units of penicillin G. Completeness of adrenalectomy was assessed in 2 ways: (a) after killing the rats at end of experiment, they were carefully examined with a magnifying lens for adrenal remnants; and (b) 17 rats of the same strain, age and weight were adreno-ovariectomized and supplied with tap water instead of 1% saline. Death occurred in 16 rats on the following days after surgery: one rat on day 7, 2 on day 9, one on day 10, 3 on day 11, 3 on day 12, 2 on day 13, and one each on days 14, 15, 17 and 19. The remaining rat was killed on day 24, and one adrenal was found which had not been completely removed. The sella turcica of all hypophysectomized rats was examined for pituitary fragments at end of experiment. Only rats with complete pituitary removal are included in the results.

Twenty-four hours after adreno-ovariectomy, all rats were treated for 10 days as shown in Table I. Hormones were injected thrice daily at intervals of approximately 6 hours between 9 A. M. and 9 P. M. Bovine STH‡ and ovine prolactin‡ were dissolved in distilled water and anterior pituitary powder§ was suspended in physiological saline. All rats were fed *ad libitum* on Wayne Lab-Blox pellets supplemented with canned Armour's Dash dog food, and were given 1% saline to drink. Slices of oranges and 5% glucose in drinking water were made available to all hypophysectomized rats. Body weights of all rats were recorded at beginning and end of each treatment.

The rats were killed on the day following last injection, and both inguinal mammary pads were dissected free, spread flat on cork and fixed in Bouin's fluid. One mammary pad from each animal was stained by a standard procedure(6), and rated for development according to the following scale:

I = Few ducts; few or no end buds
II = Moderate duct growth; moderate

‡ Donated by Endocrinology Study section, NIH.
§ Supplied through courtesy of Dr. J. D. Fisher, Armour Pharmaceutical Co., Kankakee, Ill.

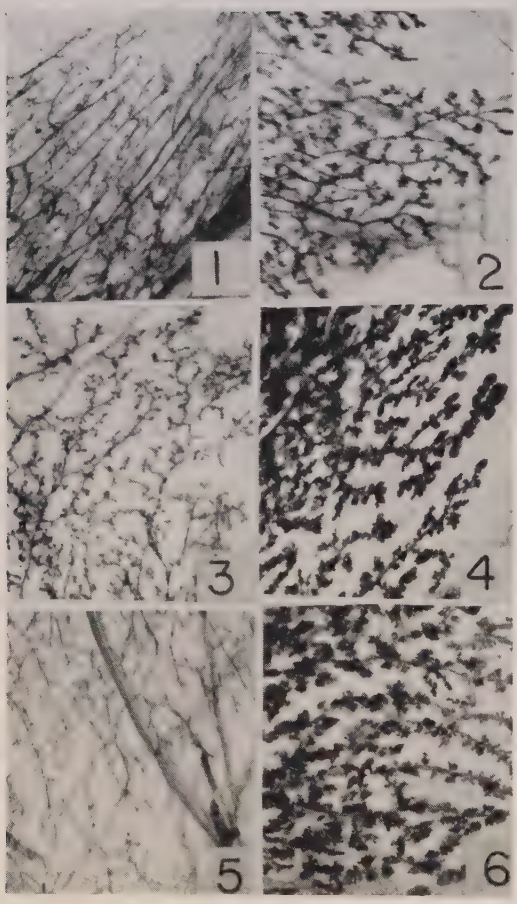

FIG. 1-6: Photographs of whole mounts of mammary glands from rats treated 3× daily for 10 days. × 7.5.

Fig. 1-4: Adreno-ovariectomized rats.

FIG. 1. 0.1 ml saline. Moderate duct development and few end buds.

FIG. 2. 2 mg STH. Extensive duct growth with many end buds.

FIG. 3. 30 I.U. prolactin. Moderate duct growth with many end buds.

FIG. 4. 1.33 mg STH and 20 I.U. prolactin. Extensive duct and dense lobulo-alveolar development.

Fig. 5-6: Adreno-ovariectomized-hypophysectomized rats.

FIG. 5. 0.1 ml saline. Few ducts with little branching.

FIG. 6. 1.33 mg STH and 20 I.U. prolactin, Extensive duct and lobulo-alveolar development.

number of end buds

III = Numerous ducts and branches; many end buds

IV = Numerous ducts and branches; moderate lobulo-alveolar (L-A) growth

V = Numerous ducts and branches with dense L-A growth, as in mid or late pregnancy.

Results. The data are summarized in Table I. In doubly operated rats, mammary glands from saline treated controls (Group 1) consisted of moderately developed ducts with few end buds (Fig. 1). Treatment with STH (Group 2) induced extensive duct growth with many branches and end buds but no L-A growth (Fig. 2). Prolactin (Group 3) also induced duct growth with many branches and end buds (Fig. 3), and 3 out of 7 rats showed limited L-A development. Combined treatment with STH and prolactin (Group 4) induced extensive duct growth and dense L-A development comparable to that seen in rats after mid or in late pregnancy (Fig. 4). Mammary growth produced by anterior pituitary powder (Group 5) did not equal that produced by combined STH and prolactin treatment, but most rats showed moderate L-A development. In triply operated rats, mammary glands from control animals (Group 6) consisted essentially of small ducts with little branching and few or no end buds (Fig. 5). Combined treatment with STH and prolactin (Group 7) produced extensive duct growth and branching and moderate L-A development (Fig. 6).

Doubly or triply operated controls gained very little in average body weight during treatment, while rats given STH or STH and prolactin gained an average of 41-43 g each. Prolactin and anterior pituitary powder produced average body weight gains of 14 g and 20 g, respectively.

Discussion. In previous studies with triply operated rats, administrations of estrogen and progesterone or adrenal corticoids were needed in addition to STH and prolactin to induce lobulo-alveolar (L-A) growth(2). The results of the present experiment show that in doubly or triply operated rats combined treatment with bovine STH and ovine prolactin, or bovine anterior pituitary powder alone, can induce L-A development. Our results are therefore in agreement with those of Clifton and Furth(5) who found good L-A development in adreno-gonadectomized male rats implanted with "mammotropic" pituitary tumors. The procedures used here are

believed to account to a large degree for the present findings. Thus, prolactin and STH or whole anterior pituitary powder were injected thrice instead of only once daily; injections were begun the day after surgery; and relatively large doses of these hormones were used. Also, our rats were mature females, 3 to 4 months old, whereas Lyons *et al.*(2) employed mainly young rats.

Ovariectomy and adrenalectomy appeared to be complete in these rats. The 16 adrenalectomized rats not given saline died within 19 days. Since injections were begun 24 hours after removing the ovaries and adrenals, the possibility cannot be completely excluded that small amounts of steroid hormones may have been present during the first few days of treatment. It is doubtful however, that this would have been sufficient to synergize with the anterior pituitary hormones to induce full L-A growth. Clifton and Furth(5) implanted pituitary tumors in rats 4 to 16 days after adreno-gonadectomy and still obtained good L-A development.

The present results cannot be interpreted to mean that ovarian and adrenal cortical hormones have no role in normal mammary development in rats. Estrogen and progesterone have been shown to induce full mammary growth in intact or ovariectomized rats (1) and to synergize with prolactin and STH in triply operated rats(2). However, the anterior pituitary appears to be of primary importance in mammary growth in the rat, since anterior pituitary hormones alone induced full mammary development in the absence of ovaries and adrenals, whereas ovarian or adrenal cortical steroids have little or no effect on mammary growth in the absence of the anterior pituitary(1-3).

Summary. 1. Mature virgin female Carworth rats of the CFN strain were adreno-ovariectomized and were then injected thrice daily for 10 days with saline, 2 mg bovine STH, 30 I.U. ovine prolactin, 20 mg bovine anterior pituitary powder, or with a combination of 1.33 mg bovine STH and 20 I.U. ovine prolactin. Treatment with STH and prolactin together or with anterior pituitary powder induced moderate to extensive mammary duct and lobulo-alveolar growth, while treatment with prolactin and STH alone induced mainly duct and end bud development. 2. Two groups of rats were hypophysectomized and 3 days later were adreno-ovariectomized. They were then injected thrice daily for 10 days with saline or with a combination of 1.33 mg STH and 20 I.U. prolactin for 10 days. The latter treatment induced extensive duct and moderate lobulo-alveolar growth. Completeness of ovariectomy, adrenalectomy and hypophysectomy was checked in all rats upon autopsy. Sixteen additional adreno-ovariectomized rats which were given tap water failed to survive more than 19 days. 3. It is concluded that under conditions of this experiment, full mammary duct and lobulo-alveolar growth was induced by combined treatment with bovine STH and ovine prolactin in the absence of ovaries and adrenals.

Addendum. Histological examination of stained mammary sections from adreno-ovariectomized rats treated with prolactin and STH failed to reveal any secretory activity.

The authors are indebted to Mr. T. F. Hopkins for performing the hypophysectomies and to Mr. C. S. Nicoll for the photographs.

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Humoral Regulation of Erythropoiesis VII. Shortened Survival of Erythrocytes Produced by Erythropoietine or Severe Anemia. (26784)

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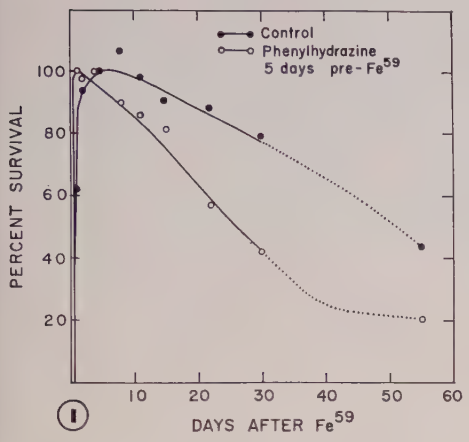
After severe blood loss or destruction of the entire red cell mass with phenylhydrazine, the hemoglobin in the rat will return to pretreatment levels within 10-15 days. Thereafter there is continued production at about normal levels as estimated by reticulocyte counts and iron incorporation studies. At this time in the phenylhydrazine treated rat there are no cells older than 15 days so that there is no senescent loss; senescent loss must also be strikingly reduced in the bled animals. Polycythemia, however, does not ensue. These observations suggest that the mean life span of red cells formed in response to severe anemia is shortened. A shortened life span of red cells produced in response to acute blood loss has been reported by Berlin and Lotz(1).

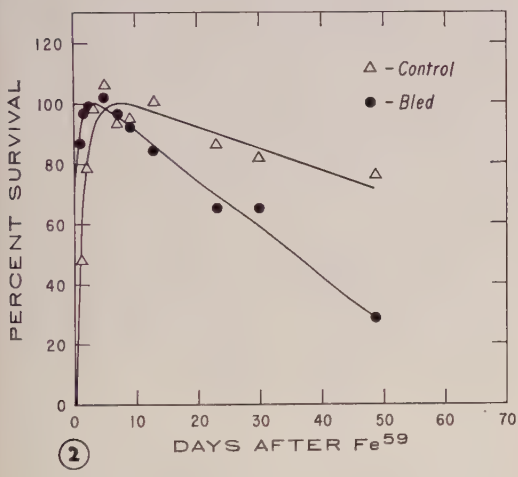
Erythropoietine is believed to be the stimulus for increased red cell production in both bled and phenylhydrazine treated animals. It therefore seemed likely that cells produced in response to erythropoietine would also have a shortened red cell survival. Data to document this hypothesis are presented here.

Materials and methods. Female Sprague-Dawley rats were used throughout; test animals weighed 135-160 g at beginning of the experiment; donor rats weighed 200 g or more. Phenylhydrazine was given in doses of 4 mg/100 g on days 0, 1 and 3. In the experiments on acute blood loss, rats were bled by cardiac puncture 1.5% of body weight on days 0 and 1. The animals receiving erythropoietine were transfused with packed red cells obtained from heparinized normal blood 4 days prior to the first injection of erythropoietine; each animal was given a volume of red cells equivalent to 1.65% of body weight. In the first experiment 5 subcutaneous injections of 0.5 cc of erythropoietine containing ~ 10 C.S.(2) units were given at 12 hour intervals. In a second experiment 0.5 cc of erythropoietine containing ~ 10 C.S. units was given subcutaneously on 3 occasions 24 hours apart. Erythropoietine was obtained by alcoholic extraction of urine from a patient with congenital hypoplastic anemia.

Measurement of survival. Plasma bound Fe^{59} was injected intravenously as previously described(3); 3 hours and at daily intervals thereafter 5 mg of non-radioactive iron (Imferon) was given intramuscularly to minimize reutilization of Fe^{59}. Fifty treated and 50 control animals were randomized for each experiment. Five animals were used to determine each point. Twenty minutes prior to exsanguination washed Cr^{51} tagged normal cells were injected intravenously for blood volume determination(3). Fe^{59} and Cr^{51} counts were separated with a single channel spectrometer. Total red cell Fe^{59} activity was determined(3) and expressed as the per cent of injected dose. These values were plotted against time and per cent survival was determined using the peak Fe^{59} incorporation value as the 100% value. Red cell counts were done electronically(4); hematocrits were measured by Strumia's method(5) and reticulocytes were counted by Brecher's method(6).

Results. 1. Phenylhydrazine. Radioiron was given 2 days after the last injection of phenylhydrazine. At that time the reticulocyte count was ~ 75%, the peak reticulocytosis of 90-100% occurring on the 6th-7th day. The hematocrit was ~ 20, with a mean corpuscular volume (MCV) of 100 μ^3 (normal ~ 55 μ^3). The survival of the cells tagged at this time was clearly shortened (Fig. 1). In a second experiment, Fe^{59} was injected on the 10th day when the hematocrit was ~ 40 and the reticulocytes ~ 3.0%. Degree of shortening of red cell survival was less than when cells were labeled on the 5th day; for example on the 20th day after Fe^{59} there was ~ 80% survival compared with





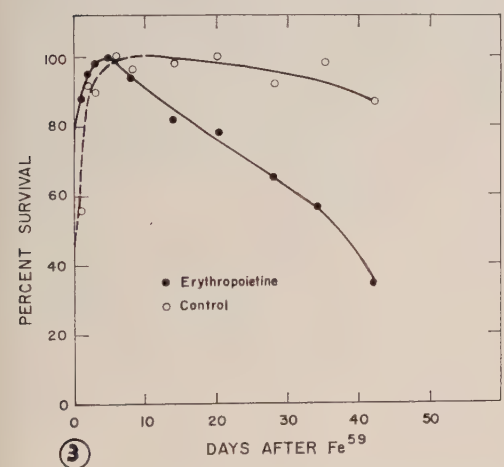


FIG. 1. Survival of red cells produced in response to phenylhydrazine induced anemia. Radioiron was given 5 days after first inj. of phenylhydrazine.

FIG. 2. Survival of red cells produced in response to acute bleeding.

~ 60% survival in the group given Fe^{59} on the 5th day after phenylhydrazine and ~ 90% in the control group.

2. Bled Animals. The animals were injected with Fe^{59} 48 hours after the initial bleeding. At this time the hematocrit was ~ 25, the MCV ~ 61 μ^3 and reticulocytes ~ 10%. The following day the reticulocytes were ~ 14%; peak reticulocytosis of ~ 21% was reached on the 6th day after bleeding. The period of survival of the red cells produced in response to this severe blood loss was also shortened (Fig. 2).

3. Erythropoietine Treated Group. At time of injection of erythropoietine the hematocrit of recipient animals was ~ 55 (normal 40-44) and reticulocytes 0.1-0.2% (normal 1-3%). Five injections of erythropoietine produced a reticulocytosis of 10-12% on the 4th day, an increase in hematocrit to ~ 60 and in the MCV to ~ 61 μ^3. After erythropoietine was stopped, reticulocyte values returned to 0.1-0.2%. Thereafter the hematocrit and blood volume slowly decreased until they returned to normal at ~ the 28th day, when the reticulocyte count was normal. Radioiron was given 12 hours after the last injection of erythropoietine. The survival of cells tagged at this time was significantly shortened (Fig. 3). In another experiment 3 injections of erythropoietine were given at 24 hour intervals and Fe^{59} was given 24 hours after the last injection of erythropoietine. There was a reticulocytosis of ~ 4% associated with a borderline shortening of red cell life span.

Discussion. There was a shortened life span of red cells formed in response to severe anemia, whether due to phenylhydrazine or blood loss. A similar effect was demonstrated with erythropoietine, which is known to be in part responsible for the increased red cell production in severe anemia. The use of hypertransfused animals, which reduced normal production to a minimum, permitted us to study primarily the life span of cells produced in response to erythropoietine. Values for the control animals were comparable to

FIG. 3. Survival of red cells produced in response to erythropoietine.

those observed by Berlin and Lotz with C^{14} glycine(1) and Harriss and Belcher with Fe^{95} followed by Imferon(7). There was a variable degree of random destruction between experiments in control groups but the curves for the experimental groups shown in Fig. 1-3 were well outside the normal range observed in these and 5 other experiments in our laboratory.

Van Dyke and Berlin(8) have previously published data which led them to conclude that cells produced in response to erythropoietine had a normal life span. The apparent discrepancy between their conclusions and ours might be explained by a dose effect. However, their data are not inconsistent with ours. They induced polycythemia by administration of erythropoietine over a 10 day period, hematocrits rising from 40 to 55. Midway in the period of treatment, C^{14} labeled glycine was given, and *specific activity of heme* determined thereafter. Specific activity reached a maximum by the 10th day and thereafter remained constant for the next several weeks. This led them to conclude that the life span of the cells was normal. In fact after erythropoietine was discontinued, red cell production should have stopped due to the induced polycythemia. At this point the labeled cells produced in response to erythropoietine would have been 0-10 days old. Senescent loss of older cells formed prior to administration of erythropoietine should have continued. Due to the polycythemia-induced suppression of erythropoiesis, these cells would not be replaced. If the survival of labeled cells was normal, the loss of cells with *unlabeled heme* without replacement would result in a constant increase in the proportion of labeled cells and hence an increase in the *specific activity* of heme. When erythropoiesis is suppressed the specific activity of heme can remain constant only when loss of labeled cells is equal to loss of unlabeled cells. This must have occurred in the studies reported by Van Dyke and Berlin and implies a shortening of the life span of cells formed in response to erythropoietine.

Erythropoietine acts primarily through differentiation of stem cells into the erythroid compartment(9,10). After large doses of erythroypoietine, the magnitude and rapidity of the response suggest that the differentiated cells either skip divisions or that generation times are shortened(11). In either event the result is the release of a "younger" reticulocyte which is much larger than the normal reticulocyte(12,13). In the phenylhydrazine treated animals, for example, MCV was 100 μ^3 in contrast to the normal of 55 μ^3. In the bled and erythropoietine treated groups the shift in MCV was less marked but normal cells were still present in these animals. To shift the MCV from 55 μ^3 to 61 μ^3 by introducing a population of macrocvytes amounting to 10% of the total population requires that these macrocytes have an average MCV approaching 100 μ^3. Size distribution studies bear out the increased size of these newly formed cells(12). It may be that these macrocytes have a shortened cell survival due to their size and shape much as do spherocytes in hereditary spherocytosis. The rapid rate of formation of cells may also lead to other defects which affect life span.

It is not possible to state whether production of the large cells is a dose-dependent phenomenon. The dose relationship is being investigated. If erythropoietine invariably results in production of large cells, this strongly supports our argument that erythropoiesis is not under a single control. Though data presented on the effect of high doses of erythropoietine do not offer conclusive proof they are in agreement with the hypothesis that red cell production is regulated by more than one mechanism, erythropoietine being a panic mechanism(14).

Summary. Red cells formed in response to erythropoietine or severe anemia have a substantially shortened life span. The possibility is proposed that this is related to the macrocytic character of the cells. The results are in agreement with the hypothesis that erythropoietine is not the sole regulator of red cell production.

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Reticulocyte Size and Erythropoietic Stimulation. (26785)

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It is generally accepted that reticulocytes are larger than mature red cells(1). The manner in which the oversized reticulocyte matures into a red cell of normal size has not been defined. Recently, Stohlman found that red cells produced during recovery from severe phenylhydrazine anemia have a markedly shortened life span(2). The mean corpuscular volume of the reticulocytes so produced was nearly twice that of normal mature red cells. The results suggested that these macrocytic cells are replaced by cells of more normal size. This thesis was confirmed in the present studies by measuring the distribution of cell sizes; the results indicate that a correlation exists between rapidity of red cell regeneration and size of reticulocytes.

Methods. Groups of female Sprague-Dawley rats weighing 140-160 g were injected s.c. with either 0.1 or 0.3 ml of a 2% solution of phenylhydrazine hydrochloride (PHH) in distilled water on days 0, 1, and 3 of the experiment. A third group served as controls. In one experiment, one group of animals was injected with 0.3 ml PHH on days 0, 1, and 3, then given 650 r whole body irradiation on day 4. Rats were exsanguinated by cardiac puncture at various intervals following last injection.

Mean corpuscular volumes (MCV) were determined from hematocrits measured by a micro method and red cell counts measured with the Coulter Counter(3). Red cell size distribution curves were obtained with a Coulter Counter Model B supplied with an automatic graphout. The Model B is a modification of the basic Coulter instrument which generates impulses proportional to cell volume as individual red cells pass through a 50 micra aperture. An electronic gating device of the Model B* permits discrimination of impulses of different magnitude, corresponding to different cell volumes. The graphout automatically records their frequency distribution. In the resulting graphs, the ordinate represents relative frequencies. The linear scale of the abscissa corresponds to the amplitude of the electronic impulses. The proportionality factor between amplitude of impulses and cell volume can be determined by appropriate calibration and units on the abscissa converted to μ^3. This has been done in Fig. 1. The peaks or modal values of distribution curves are, as a rule,

* Available from Coulter Electronics, Inc., Chicago, Ill.

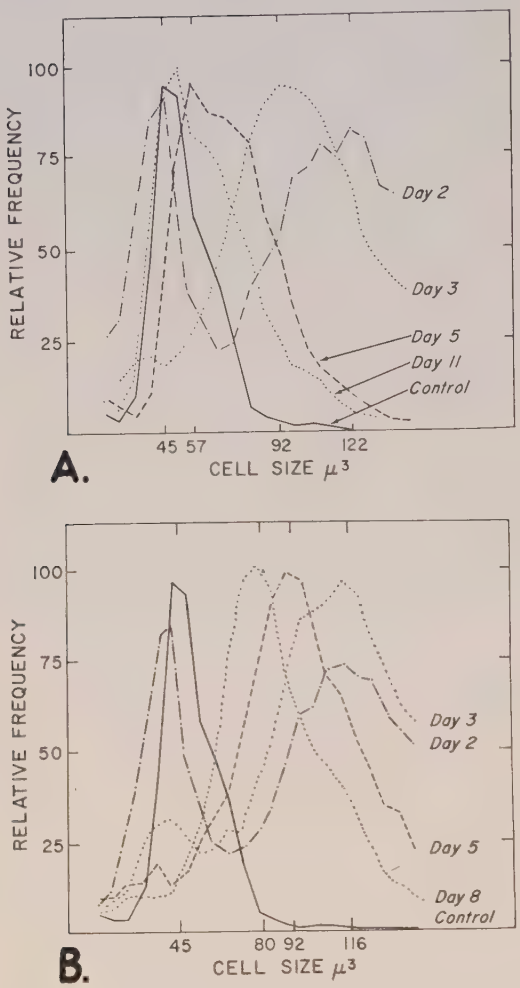

FIG. 1. Size distribution of cells produced in response to phenylhydrazine (PHH) anemia in controls (A) and irradiated rats (B). Days counted from day after last inj. of PHH. All measurements were made on red cell suspensions adjusted to approximately 200,000 cells/ml. Changes in animals' hematocrits are given in text.

lower than MCV's due to skewing of the curves to the right. MCV's can be computed from the graphs and agree within 4% with MCV's computed as ratio of hematocrit to rbc count. Detailed data on calibration and reproducibility will be published elsewhere. For counting, red cells were suspended in a mixture of 9 parts of the electrolyte and glucose mixture used in Eagle's tissue culture medium and 1 part of distilled water. Size distribution curves were the same in this solution as in plasma.

Partial separation of reticulocytes and mature cells was obtained by centrifugation. Two technics were used. In the first, whole versenated blood was spun, the plasma removed, the cells washed twice in cold Eagle's solution and recentrifuged in a Wintrobe hematocrit tube. The top and bottom layers were then sampled with a Wintrobe pipette. In the second method, whole blood was centrifuged in a 75 mm microhematocrit tube. Top and bottom layers were sampled by a smaller capillary to which plastic tubing was attached for suction, or by breaking the capillary at desired points after marking it with a file. Since only 2 lambda are needed for red cell sizing and similarly small amounts for reticulocyte counts, it was possible to obtain both from a narrow segment of the top and bottom layer respectively. Greater speed and better separation are achieved with the microhematocrit than with the Wintrobe tube. However the latter method was mandatory when additional measurements, such as MCHC, were desired.

Results. In rats given 3 doses of 0.3 ml of phenylhydrazine (PHH), the hematocrit was 26% on the day after last injection, 37% on the 3rd day and returned to a normal value of 42% by the 5th day after last injection. Reticulocytes were 40-55% on the 2nd day and 90-95% on the 3rd day. Two days after the last PHH injection, the erythrocytes were composed of 2 distinct cell populations (Fig. 1, A): cells of normal size with a modal value of 45 μ^3, and cells of more than twice normal size, the modal value being 122 μ^3. The normal sized cells were rapidly eliminated as may be seen by the marked reduction in the cohort of cells with a modal value of 45 μ^3 on the 3rd day after last PHH injection. Thereafter only the larger cells were present, but the distribution curve shifted rapidly toward normal. Modal values were 93 μ^3 on the 3rd day, 57 on the 5th day and 51 on the 11th day after PHH injection. Theoretically, the shift in modal value could be due to shrinkage of cells, selective destruction of larger cells, or production of smaller cells. Shrinkage of the red cells could be eliminated on the basis of measurements of mean corpuscular hemo-

TABLE I. Modal Values of Red Cell Size Distributions after Different Doses of PHH.

Days after PHH	Total dose, 2% PHH, ml	Peaks of size distribution, μ^3		Reticulocytes, %		
		Top	Bottom	Top	Bottom	Whole blood
1	.9	63, 98	45	99	6	45
4	.9	75, 86	86	94	87	93
5	.9	57, 75	75	71	57	64
8	.9	57	69	15	4	9
15	.9	51	75	4	0	1
1	.3	45, 69	45	93	5	15
4	.3	69	45, 69	83	20	53
5	.3	69	63	66	28	40
8	.3	51	69	41	1	9
15	.3	51	69	7	0	2
—	—	45	45	9	1	2

globin concentration (MCHC) and MCV. On the 3rd day after last PHH injection, when 90-95% of cells were reticulocytes, MCV was 100-110 μ^3 (normal ~ 55 μ^3) and MCHC was 27-30% (normal 34-36%). Shrinkage of the oversized reticulocytes to normal sized adult red cells would have resulted in an MCHC of 49-60%. No values over 36% were observed.

To determine to what extent production of smaller cells can account for the shift in cell size distribution, a number of animals were irradiated on the day after last PHH injection. Erythropoiesis was markedly suppressed. The hematocrit dropped from a normal of 40 to 45% to 17% on the 3rd day after last PHH injection and stayed approximately at this level until the 11th day. Cell size distribution in this group of animals (Fig. 1, B) was at first indistinguishable from that of the non-irradiated rats. The distribution curve again showed 2 populations with normal modal values of 45 and 116 μ^3 on the 2nd day after last PHH injection. The normal sized cells corresponding to the lower modal value virtually disappeared by the 3rd day. Subsequently, however, the modal value of the larger cells changed only slowly in the irradiated rats. It was 92 μ^3 on the 5th day and 80 μ^3 on the 8th day, where it remained until termination of the experiment 11 days after last PHH injection. Control animals irradiated without prior PHH injection had no shift in cell size distribution. Thus, suppression of erythropoiesis by irradiation greatly retarded the rapid shift in the distribution curve observed in rats with a normal erythropoietic response.

To test whether lesser degrees of hemolysis and erythropoietic stimulation would result in cells of more nearly normal size, 2 groups of rats receiving 3 doses of 0.3 ml or 0.1 ml of PHH were compared. In contrast to the double peaks of 45 and 98 μ^3 on the day after last PHH injection in the group with the higher dose, the rats that had received the lower dose had only a broadening of the curve to the right. Three days later the red cells of the high dose group had a single peak with a modal value of 86 μ^3; modal value in the low dose group was 69 μ^3. Production of cells of more nearly normal size in both groups is indicated by the modal values of 63, 51 and 48 μ^3 on days 5, 8, and 15 following the higher dose, and modal values of 63, 63, and 51 μ^3 on the same days in the low dose rats. In both groups the continued presence of larger cells was indicated by the broad tail to the right in all curves. Separate size distribution and reticulocyte counts on the top and bottom layers of centrifuged specimens provided further insight into production of cells of different sizes in the 2 dosage groups (Table I). On the day after last PHH injection, the bottom layer of cells of both groups contained predominantly surviving normal cells with a modal value of 45. The top layer contained 2 populations in both groups. Modal values were 45 and 69 μ^3 in the low dose group, and 63 and 98 μ^3 in the high dose group. In both the high and low dose group there was a gradual reversal in

TABLE II. MCV and MCHC of Red Cells in a Centrifuged Column.

Total dose 2% PHH, ml	Peaks of size distribution, μ^3		MCV, μ^3		MCHC, %	
	Top	Bottom	Top	Bottom	Top	Bottom
.9	75	92	106	109	22	29
.3	63	39, 63	83	73	29	34
—	45	45	57	57	32	34

distribution of smaller and larger cells between top and bottom layers. Reticulocytes continued to be more numerous in the top layer, in keeping with the hypothesis that the very large reticulocytes originally produced are being replaced by successive crops of reticulocytes of more normal size.

The continued predominance of reticulocytes in the top layer appeared to support the thesis that centrifugation results in layering of red cells in accordance with their age. It could be readily shown, however, that this is not always the case. When blood from patients with microcytic hypochromic anemia was examined during the first week of iron therapy, reticulocytes congregated in the middle or bottom layer after centrifugation, strongly suggesting that mean corpuscular hemoglobin concentration (MCHC) rather than age determines the position of red cells in a centrifuged specimen. To test this thesis, MCHC, MCV and size distribution were determined in the top and bottom layers of selected blood specimens of rats that had received 0.9 and 0.3 ml PHH, and of controls. MCHC was lower in the top layer in each instance, while the MCV was larger in one instance, smaller in another, and the same as the bottom layer in the third (Table II).

Discussion. The rapid replacement of the first crop of reticulocytes produced in severe phenylhydrazine anemia has been established by their shortened life span(2). These early reticulocytes, which at one time represent about 90% of all red cells, have an MCV of twice normal but an MCHC only 20% less than normal. This precludes their shrinking to cells of normal size and MCHC. It was to be expected, therefore, that early, greatly oversized reticulocytes will be replaced by cells of more normal size. This could not be established by determination of MCV alone, but was supported by measurement of cell size distribution, which demonstrated the presence of 2 populations of cells with different volumes. When frequency distribution of cell volumes was measured in different cell layers after partial separation of reticulocytes by centrifugation, a general picture of decreasing volumes of successive crops of reticulocytes was observed. It could also be demonstrated that the reticulocytes initially produced after high dosage of phenylhydrazine are almost twice normal size, whereas those produced after a lower dose are only about 1.5 times normal size. The intensity of erythropoietic stimulation thus appears to govern reticulocyte size to some degree. Skipped division may account for the larger size of more rapidly produced cells(4).

The position of cells in a centrifuged column of blood presumably is governed by the density of the cell. The present data suggest that the MCHC is the principal variant in red cell density. The sequence of events occurring after administration of PHH support this thesis. The MCHC of the very large reticulocytes produced initially (Fig. 1) was lower than that of the normal red cells. Consequently, at this time the larger cells were in the top layer after separation. The continued hemoglobin synthesis in these large reticulocytes eventually resulted in a normal MCHC. We suggest that a new crop of smaller reticulocytes with an MCHC below normal emerged at this time, accounting for the continued accumulation of reticulocytes in the top layer of centrifuged blood.

Our argument implies that eventually reticulocytes of the size of normal adult cells are produced, and that such normal sized reticulocytes replace the normal daily losses from senescence. The top and bottom layers of centrifuged normal blood have, in fact, never shown measurable differences in cell size.

However, minor degrees of excess size might escape detection unless 100% separation of reticulocytes is achieved. The best separation in normal blood with the technique used was 9% reticulocytes in the top layer and 1% in the bottom layer. (Control, Table I). Improved techniques for reticulocyte separation are needed to settle the point.

Summary. The size distribution of peripheral red blood cells of rats recovering from phenylhydrazine anemia is reported. Reticulocytes produced during the first burst of active erythroid regeneration are as much as twice the normal size; degree of macrocytosis is related to the severity of the anemia. The initial crop of oversized cells is replaced by successive crops of reticulocytes of more normal size. It is suggested that separation of reticulocytes by centrifugation depends on their density, which is determined primarily by the MCHC.

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Comparative Effects of Colchicine and Vincaleukoblastine on Bone Marrow Mitotic Activity in Syrian Hamster.* (26786)

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Orsini and Pansky(1) reported the Syrian hamster to be resistant to colchicine. They were unable to observe metaphase arrest in dividing cells in animals treated with doses of colchicine varying from 1 mg to 20 mg/kg of body weight. Since this original report by Orsini and Pansky, the Syrian hamster has been generally accepted as being resistant to colchicine(2).

Recently an alkaloid derived from *Vinca Rosea Linn.,* Vincaleukoblastine, was found to possess, like colchicine, the property of arresting cell mitosis at the metaphase stage (stathmokinetic effect)(3,4,5).

The present study was undertaken to study the comparative effects of Vincaleukoblastine and colchicine in Syrian hamsters.

Methods. Fifty Syrian hamsters† of both sexes, weighing 80-130 g, were used in these experiments. Three groups of 10 animals each were injected intraperitoneally with 1.2 mg/kg, 2.4 mg/kg and 7.2 mg/kg, respectively, of colchicine. They were sacrificed 4 hours after treatment. A fourth group of 10 hamsters was injected with 1 mg/kg of Vincaleukoblastine and also sacrificed after a period of 4 hours. A fifth group of 10 hamsters was used as a control.

Bone marrow from the femurs of all animals was collected and fixed in 70% methyl-alcohol. Preparations for microscopic study were made according to the Feulgen squash method. Scoring of mitotic figures was done by counting 2000 cells in each preparation. Survival time after intraperitoneal treatment with high doses of colchicine and Vincaleukoblastine was determined in AKR mice, WR rats, and Syrian hamsters.

Results. The results of these experiments, reported in Tables I, II, and III, indicated that, although hamsters seemed to be resistant to the general toxic effects produced by colchicine, as demonstrated by survival time, their bone marrows were only partially resistant to the stathmokinetic effect of this alkaloid. Colchicine, in a dosage of 1.2 mg/kg,

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† Dennen Animal Industries, Gloucester, Mass.

TABLE I. Bone Marrow Mitotic Index in the Normal Syrian Hamster before and after Treatment with Colchicine and Vincaleukoblastine.

No. of animals	Compound	Dosage, mg/kg	Mitotic index, %
10	—	—	13.7
10	Colchicine	1.2	15.1
10	"	2.4	38.35
10	"	7.2	38.30
10	Vincaleukoblastine	1.0	98.2

TABLE II. Survival after a Single Injection of Colchicine.

Animal	No.	Dosage, mg/kg	Survival, days 2	5	30
Mouse	5	9.6	0	0	0
Rat	5	9.6	1	1	0
Hamster	5	9.6	5	4	4
Mouse	10	19 2	0	0	0
Rat	10	19.2	0	0	0
Hamster	20	19.2	19	18	18

TABLE III. Survival after a Single Injection of Vincaleukoblastine.

Animal	No.	Dosage, mg/kg	Survival, days 2	5	30
Mouse	5	5	5	3	3
Rat	5	5	5	2	1
Hamster	5	5	5	4	2

which produced complete metaphase arrest of all dividing cells in the mouse and rat, was practically ineffective in the hamster. However, when dosage was raised to 2.4 mg/kg, an accumulation of arrested metaphases was observed and the mitotic index increased to *circa* 3 times that of the control. There was no apparent further increase in this stathmokinetic effect by increasing the dose of colchicine from 2.4 to 7.2 mg/kg. Moreover, even at a dose level of 7.2 mg/kg, colchicine did not produce metaphase arrest in all of the bone marrow cells, since some post-metaphase figures were also present. On the other hand, Vincaleukoblastine produced complete metaphase arrest. The number of accumulated metaphases in hamsters treated with Vincaleukoblastine was very high (Table I) and no post-metaphase mitotic figures were observed.

Conclusions. The data presented here indicate: (1) the Syrian hamster is equally as sensitive to the stathmokinetic effect of Vincaleukoblastine as are the mouse and rat; (2) Syrian hamster bone marrow cells, ***in vivo,*** are only partially resistant to colchicine.

Summary. The effect of Vincaleukoblastine and colchicine on mitotic activity of bone marrow cells was studied in the Syrian hamster. Vincaleukoblastine at a dosage of 1 mg/kg, administered intraperitoneally, caused metaphase arrest of all dividing cells. Colchicine, at a dosage of 1.2 mg/kg, which caused effective metaphase arrest of dividing cells in mice, rats, and other animals, did not demonstrate stathmokinetic effect in hamsters. A single injection of 2.4 mg/kg of colchicine caused partial metaphase arrest. There was no apparent increase in the stathmokinetic effect by increasing the dose of colchicine from 2.4 to 7.2 mg/kg.

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Effects of Anterior Pituitary Preparations on Mammary Gland Growth in Mouse.* (26787)

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Previous comparisons of lactogenic and mammogenic potencies of various pituitary preparations have indicated extreme differences in relative amounts of these hormones when assayed in mice(1). Recent studies have indicated that it is possible to extract fractions with fair mammogenic activity when injected with estradiol benzoate (EB) in the ovariectomized mouse, but possessing little or no lactogenic activity(2). Present report deals with mammogenic effectiveness of a number of crude and purified pituitary extracts, including one newer mammogenic fraction extracted by improved techniques.

Materials and methods. Purified FSH, lactogen and growth hormone (GH) were obtained from the Endocrinology Study Section, N.I.H. Anterior pituitary powder (AP) was obtained by washing frozen bovine anterior pituitary slices several times with cold acetone, followed by ether extraction. Resulting dry, fat-free slices were then ground to a fine powder in a Wiley Mill. Initial residue (IR) was prepared as described previously(2). A concentrated mammogenic preparation (mammogen "C") extracted by stirring one part initial residue twice with 15 parts (by weight) distilled water at approximately 29°C for 2-3 hours, centrifuging and saving clear supernatants. Supernatants were pooled and made up to 60% by addition of 95% ethanol. After adjusting pH to 12-13 by addition of 2N NaOH, solution was chilled by placing in deep freeze (–10°C) overnight. A fine precipitate formed either immediately (during chilling) or on standing thereafter at room temperature for several days. A Whatman No. 50 filter paper over a Buchner funnel was used to recover the precipitate, after which it was dried by washing with acetone. Resulting precipitate was crystalline, white, apparently salt-free, and readily soluble in distilled H_2O. Wilhelmi preparations XIX-36-A, XIX-36-B, XIX-36-C, XIX-34-C1 were prepared as follows (3). XIX-36-A represented dialyzed, lyophilized water extract of whole ground fresh bovine anterior pituitary lobes at pH 5.5. Principal component reported was FSH plus small amount of LH. XIX-36-B was prepared from residue of XIX-36-A resuspended in 0.1 M ammonium sulfate, pH 4, reextracted, dialyzed and lyophilized. Fraction was rich in TSH and LH. XIX-36-C was prepared from residue of XIX-36-B, resuspended in 0.25 M ammonium sulfate, pH 7.5, reextracted, dialyzed and lyophilized. Fraction rich in GH. XIX-34-C1 was final residue, and contained primarily lactogenic hormone.

Groups of mature female ovariectomized Webster-Swiss mice received subcutaneous injections beginning day 14 post-operatively. EB and progesterone (P) were dissolved in olive oil carrier in concentrations such that each animal received 0.1 ml oil carrier daily for 19 days. Pituitary preparations were suspended in a water carrier and injected separately from the synergizing dose of 1 μg EB injected daily.

On day 20, animals were sacrificed and 7 mammary glands of each mouse were taken for desoxyribosenucleic acid (DNA) determination as described previously(4).

Results. Crude bovine AP, when injected with EB, produced greatest mammary gland growth of any preparations in dose injected but did not produce growth equivalent to pregnancy as measured by total DNA (Table I). IR produced a greater mean total DNA than either EB alone or EB plus P, but did not differ significantly from either. Mammogen "C" produced significantly

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† Postdoctoral Fellow of Nat. Cancer Inst.

TABLE I. Anterior Pituitary Preparations on Ovariectomized Mouse Mammary Gland Growth.

Treatment	No. of mice	Body wt mean, g	DFFT,* mg	DNA, μg/mg DFFT	Total DNA, mg	DNA/100 g BW, mg
Castrate control	10	33.9	73.3	27.7	2.03	6.00 ± .48
EB, 1 μg	10	30.1	85.1	26.5	2.26	7.29 ± .38
EB, 1 μg + P, 3 mg	9	30.4	92.2	26.8	2.47	8.43 ± .35
18-days pregnant†	28	37.4	123.5	52.0	6.22	16.90 ± .69
EB, 1 μg + AP, 4 mg	10	32.2	103.3	33.9	3.51	10.90 ±1.08
Idem + IR, 4 mg	9	29.5	92.5	28.1	2.60	8.80 ± .72
" + GH, 2 mg	9	33.4	96.6	21.7	2.10	6.27 ± .40
" + FSH, 2 mg	9	30.1	72.1	29.0	2.09	6.95 ± .84
" + lactogen, 1 mg	9	33.1	99.5	27.3	2.72	8.22 ± .61
" + mammogen "C," .02 mg	10	33.3	98.3	29.7	2.92	8.76 ± .34
" + XIX-34-C1, 4 mg	11	36.0	73.6	37.8	2.78	7.93 ± .33
" + XIX-36-A, 4 mg	9	29.6	80.2	25.4	2.04	6.89 ± .47
" + XIX-36-B, 4 mg	8	34.2	77.8	31.9	2.48	7.25 ± .37
" + XIX-36-C, 4 mg	11	33.0	92.7	26.3	2.43	7.38 ± .32

* Dry, fat-free tissue.

† Data from (6).

greater amounts of total DNA than did EB alone ($P < .01$) but only produced slightly greater mean total DNA than EB plus P. Lactogenic preparation plus EB produced greater mean DNA than either EB alone or EB plus P, although significant difference was marked by high variation in this group. GH, FSH and Wilhelmi preparations were generally ineffective in production of mammary gland growth with the exception of XIX-36-C1, containing lactogen. Total DNA produced by this preparation plus EB approached significance ($P < .08$) compared to DNA produced by injection of EB alone.

Whole mount observation indicated lobule-alveolar development in mice treated with EB plus P, and EB plus AP, IR, lactogenic preparation, mammogen "C," and XIX-34-C1.

Discussion. Previous studies in mice(2) have indicated that IR, obtained from acetone dried bovine AP after known hormones are removed produced significant mammary gland growth in mouse, when injected with synergizing dose of EB. Present study confirms these findings, and points to newer method of extraction of pituitary mammogen from IR. Mammogen "C," which produced lobule-alveolar growth when injected in very small quantity (.02 mg) with a synergizing dose of EB, had relative mammogen potency not dissimilar to mammogen "A" and "B" (2), but was apparently soluble in distilled water and salt-free (indicating less partial denaturation or contamination as in previous preparations). On basis of these data, it would appear that at least one pituitary mammogen effective in mouse is left in pituitary residue after extraction of known pituitary hormones.

Purified FSH would not be expected to produce mammary gland growth in the ovariectomized animal, except by reason of possible contamination by a pituitary mammogen. In present study, no significant lobule-alveolar growth was obtained when large amounts of crude FSH extract (Wilhelmi preparation XIX-36-A) or more purified FSH were injected into ovariectomized mouse with synergizing dose of EB, indicating mammogen is not extracted by technics employed in extraction of FSH.

Crude and purified preparations of growth hormone, when injected with EB, produced no lobule-alveolar growth, confirming previous studies using hypophysectomized C_3H/He Crgl mice(5). Higher doses should have produced noticeable mammary gland development if contamination of these fractions by mammogenic factor existed.

Summary. Mammogenic potencies of a number of crude and purified anterior pituitary fractions were determined by estimation of mammary gland desoxyribosenucleic acid (DNA) in treated ovariectomized mice. Crude bovine AP, initial residue (IR) obtained by depleting AP of known hormones,

a newly developed mammogen extracted from IR (mammogen "C") and lactogenic preparation produced lobule-alveolar development. Extracted mammogen "C" was white, crystalline, apparently salt-free and water soluble. Crude and purified FSH and growth hormone exhibited little or no mammogenic effects when injected with a synergizing dose of estradiol benzoate in ovariectomized mouse.

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Ingestion of Latex Particles by Chicken Fibroblasts in Tissue Culture.* (26788)

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The ingestion of foreign particles by certain cell types both in intact tissue and in tissue culture has been studied extensively and generally referred to as phagocytosis. Cells of the phagocytic type include mammalian granular leucocytes and those of mesoblastic origin commonly called macrophages. These cells not only can ingest bacteria, cell debris and protozoa but also may ingest smaller particles of a colloidal size, this latter phenomenon being called *ultraphagocytosis* or *colloidopexy*. The precise mechanisms of the phagocytic process are not entirely clear and some variations in the manner by which particles are taken in by cells have been described. Thus, in some cases particles appear in cell vacuoles and in others such vacuoles are not seen. Pinocytosis, or "cell drinking," is a second method by which the cell may take in material from its environment. Initially described by Lewis as a mechanism by which fluid enters cells(1), the studies of Mast and Doyle(2), Holter and Marshall(3) and Chapman-Andresen and Holter(4) indicate various materials may enter the cell in this manner. Palade has described a vesicular component of endothelial cells which functions in the pinocytotic process(5) and De Robertis and Bennett(6) have described a similar component for nervous tissue cells. Ferritin molecules are ingested by endothelial cells by the pinocytotic process as reported by Wissig(7), and small (0.014 μ) colloidal gold particles have been reported to be taken in by HeLa cells *in vitro* by Harford *et al.*(8).

No electron microscopic studies have been published of ingestion of foreign inert particles by tissue cells of the fibroblastic type. The present study presents results in which cell uptake of polystyrene latex particles having a diameter of 0.264 μ is demonstrated in tissue cultures of chicken fibroblasts examined by electron microscopy.

Materials and methods. Polystyrene latex suspensions (PSL). Polystyrene latex (PSL) was obtained from Dow Chemical Corp., Midland, Mich., run No. LS-057-A. This lot had a particle size of 0.264 μ and a standard deviation of 0.0060 μ in 577 measurements. Stock suspension of PSL was made in distilled water. PSL content of this suspension determined by direct sedimentation with technics described previously(9,10) was found to be 2.3×10^9 PSL particles/ml. PSL was then diluted further in tissue culture media to obtain the final concentration of inoculum.

Chicken fibroblast cultures. Chicken fibroblast cell suspensions were prepared by trypsinizing tissue of 9-10 day old chicken embryos. These cells were placed in large Rous or T-60 flasks and growth observed

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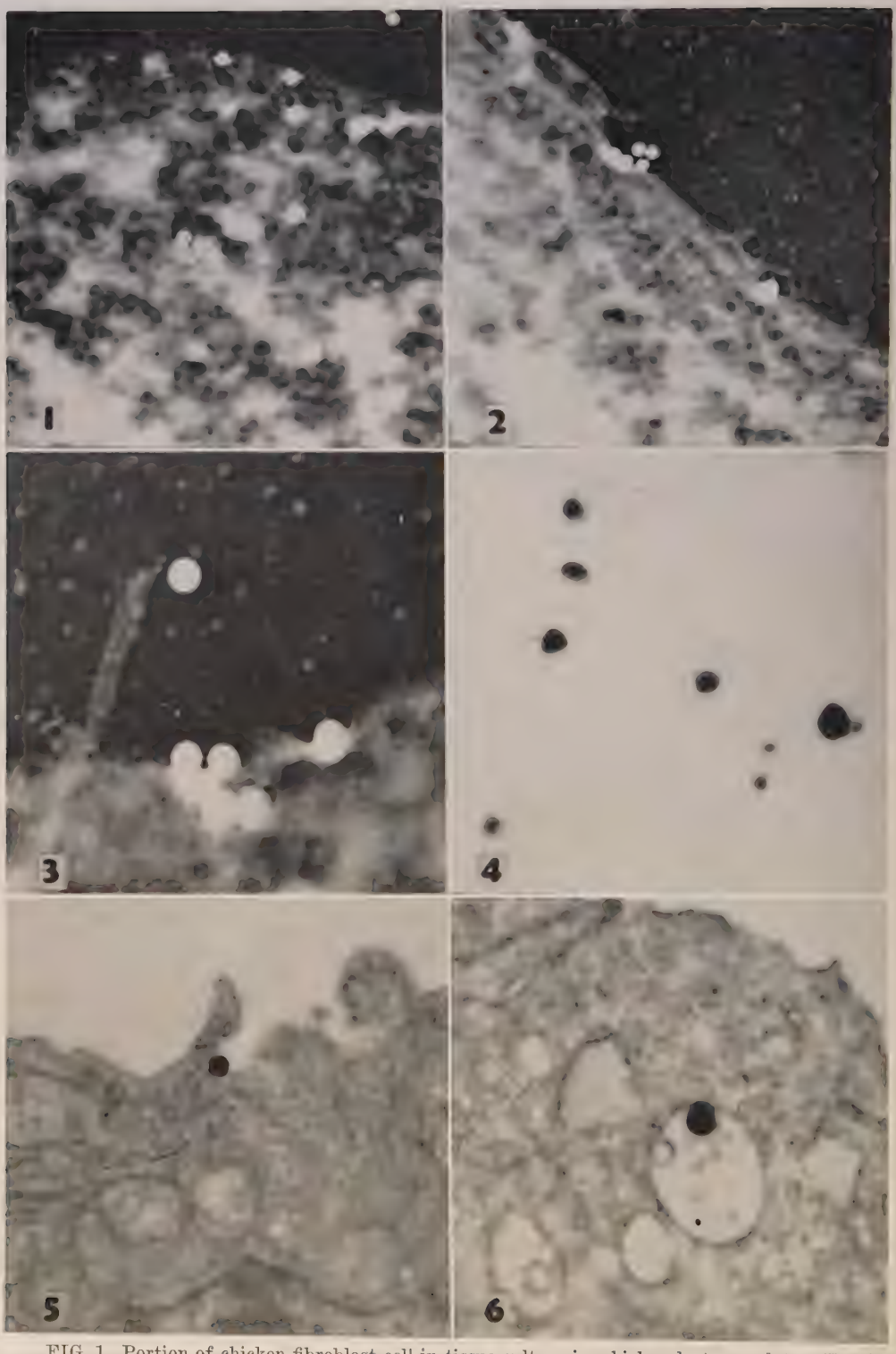

FIG. 1. Portion of chicken fibroblast cell in tissue culture in which polystyrene latex (PSL) particles are seen entering and within the cell. Chromium shadowed. × 7,500.

until monolayers had formed. The cell growth was then trypsinized from the flasks and a suspension diluted to contain 1.5 million cells. This suspension was pipetted into Leighton tubes containing carbon-collodion coated stainless steel grids. PSL was then added in ratios of 50:1 or less latex to cell. Grid mounts were removed, fixed and shadowed as described.

In some instances flask cultures of chicken fibroblasts in a monolayer were inoculated with PSL also in a ratio of 50:1. At various intervals after inoculation of PSL, the cells were trypsinized off the glass, washed 2-3 times and sedimented into a loose pellet. This pellet was then fixed with osmium tetroxide, embedded in araldite and sectioned with a Servall Porter-Blum microtome. Micrographs were taken with an RCA-EML electron microscope.

Results. In the initial experiments, PSL was added to chicken fibroblast cultures and the whole cell mounts removed at the 1 and 30 minute periods showed only a rare particle which appeared to be intracellular. However, cultures removed at the 1 and 2 hour periods or later showed many particles at various stages of entry into the fibroblast as well as large collections of PSL particles in the interior of the cell (Fig. 1, 2, 3). The general condition of cells exposed to polystyrene and observed for periods of 1-4 days showed no changes in their general morphology. Specifically addition of latex to the cultures failed to induce "toxic" changes in the cells.

An additional observation was the finding in whole cell preparations of small spikes of processes on the surface of the PSL particles in the process of engulfment. In some cases where several PSL particles were being engulfed as a group these spikes interconnected these particles. A few particles had very large spikes about 40 mμ in width (Fig. 3); others were considerably smaller. Most PSL balls entering cells showed these processes and none of the extracellular particles or particles not directly or indirectly in contact with the cell showed spikes of any size.

To help resolve the question as to whether the particles observed actually were within the cell, or rather were on top or under it, the following experiments were performed. The cell monolayer was exposed to PSL for 2 hours, trypsinized, washed several times and this cell suspension inoculated into Leighton tubes to allow the cells to reattach to the coated grid surface. These cells were observed until most had put out processes at which time the grid mounts were removed, fixed and shadowed. Photographs of these cells showed a few PSL particles in the processes. Many particles consistent with PSL size and density were seen indistinctly in the interior of many cells. Conversely, no extracellular PSL was observed in any of these preparations indicating that all removable PSL had been washed away leaving only those particles which were inside the cell or very firmly attached to it. In addition, PSL particles sprayed onto fixed cells showed very clear margins and well defined shadows. Thus, the appearance of PSL particles lying on top of cells is quite different from those intracellular particles observed in Fig. 1, 2, & 3.

Various stages of progression of PSL into the cell could be observed. The particles showed no predilection for any particular part of the fibroblast and PSL was observed entering the smooth wall of the cell body as well as the wall of cell processes. Interestingly, out of several hundred cells examined no PSL particles were found entering a microvillus. PSL particles initially appeared to be pressed against the cell wall and in the

FIG. 2. A single PSL particle with a large spike. Also seen is a group of 5 PSL particles in an early stage of ingestion. Chromium shadowed. × 7,500.

FIG. 3. Another group of 5 PSL particles enclosed in a pocket in the wall of a cell. A solitary particle nearby has a very thin spike. Chromium shadowed. × 18,000.

FIG. 4. PSL particles suspended in agar, fixed, embedded in methacrylate and sectioned. There is a varying degree of distortion of PSL particles. × 18,000.

FIG. 5. Early ingestion stage of PSL by chicken fibroblast. Cell culture fixed in buffered osmium and embedded in araldite. × 18,000.

FIG. 6. This micrograph is typical of many PSL particles ingested by chicken fibroblasts in tissue culture. × 18,000.

next stage the cell wall invaginated and the particle was surrounded by the cell wall (Fig. 2 & 3). As the particle was taken deeper into the cytoplasm the wall closed off forming a vacuole. Not all cells appeared to ingest PSL and of those that did some contained far larger numbers than others. In most instances where the PSL particle was definitely within the cell a vacuole could be seen, but in a few instances no vacuole was visible.

PSL in ultrathin sections of chicken fibroblasts. To demonstrate further the uptake of PSL by chicken fibroblasts, cell cultures were exposed to PSL, the cultures then washed, the cells removed by trypsinization, fixed and embedded for thin sectioning. To aid in identification of PSL in sections a heavy suspension of PSL was embedded in 2% agar, fixed and embedded for thin sectioning. Fig. 4 demonstrates the appearance of the PSL in sections. Some distortion of the usual spherical shape occurred and in some cases partial dissolution of PSL was found.

PSL was demonstrated readily in sections of chicken fibroblasts (Fig. 5 & 6) and the particles were usually located in cell vacuoles.

Rate of adsorption of PSL by chicken fibroblasts. In an effort to determine the rate at which PSL was taken up by the fibroblasts the following experiments were done. PSL was added to groups of T-60 tissue culture flasks containing 12 to 30 million cells per flask. Amount of PSL was determined by sedimentation counting(9,10) and concentration per flask adjusted to give a ratio of PSL/cells of 20:1, 5:1 and 3:1. PSL was also inoculated into cell free media for a control. Samples of supernate from each flask were removed at 1 minute, 30 minutes and 2, 4, 7, and 24 hours, pooled and PSL particle counts made on each pool. Each pool represents aliquots from 5 T-60 flasks.

Table I gives results of these experiments. No significant adsorption of PSL could be demonstrated, however, if 15% or less were removed it would not be detectable since this is the standard deviation of this method(9).

TABLE I. Rate of Removal of Polystyrene Latex (PSL) Particles from Tissue Culture Media by Chicken Fibroblasts.

Interval after addition of PSL to culture	PSL content of tissue culture media $\times 10^7$ PSL/cell ratios*		
	20:1	5:1	3:1
Media control	7.0	7.0	4.0
1 min.	6.5	6.2	3.6
1 hr	7.1	6.5	—
2 "	7.0	6.4	3.3
4 "	6.7	6.3	3.6
7 "	7.2	6.4	—
24 "	6.5	6.0	3.2

* Ratios calculated to result after adding a determined number of PSL particles to flasks of cells. Flask population estimated from cell counts of replicate flasks in same experiment.

The difference in counts between media control and culture samples was not significant. As PSL obviously is taken in by some cells based on visual examination, one can conclude that the overall rate of uptake is relatively low. This could hardly be otherwise since the cells in the monolayer would not be in direct contact with most of the PSL in suspension in the media. Measured uptake of PSL might be demonstrable if the PSL/cell ratio could be reduced to well below 1:1 but this has not been possible to date. The 3:1 ratio was close to the minimum PSL which could be added for accurate sedimentation counting.

Discussion. The present studies clearly demonstrate the capacity of polystyrene latex particles to enter the chicken fibroblast *in vitro* but the mechanisms involved are obscure. Primarily, the question raised is whether the process described represents phagocytosis by a cell generally considered non-phagocytic or pinocytosis in which the PSL particle is taken in during the drinking process. The invagination of the cell wall and vacuole formation surrounding the PSL particles would suggest a pinocytotic process as originally described by Lewis and since investigated by various workers. Palade(5) and Bennett(11) propose a process of "membrane flow and membrane vesiculation" which appears applicable to the present findings. This suggests that particles engaged by the cell wall (by mechanisms unknown)

are trapped in a recess formed as the wall slides around it.

It is of interest that a particle as large as the PSL used is taken into the cell by an apparently pinocytotic process. PSL of 0.264 μ diameter was used because this size is almost identical to that of vaccinia virus (.230 $\times$ .260 μ). In studies of virus particle entry into the cell a model system was sought whereby inert (PSL) and biologically active (vaccinia) material could be compared. It was thought that the inert PSL would remain extracellular but obviously, as the present experiments show, it does not. If the chicken fibroblast can ingest, probably by pinocytosis, PSL particles, it would seem likely that the entry of other particles, including viral, can occur by the same process (*e.g.* pinocytosis). The point of greatest importance, then, would not be whether a particle of 0.264 μ or less can enter a cell but what further activity the particle can stimulate after it becomes intracellular.

The PSL used in the present experiments is larger than most colloidal particles but certainly small enough so that the particles do not settle out over a period of days. PSL added to the media used in these experiments and allowed to stand showed no significant sedimentation after 2 weeks. Moreover, PSL sedimented from media showed no unusual amount of clumping. In the PSL adsorption experiments, therefore, it seems certain that few of the particles settle out during the short period of time involved. Rate of PSL uptake indicates that of the total number inoculated less than 15% are removed from the supernate fluid. Thus, although many PSL particles are seen within some cells this represents only a small portion of the whole number of particles exposed to the culture. Evidence of this type suggests that ingestion of these particles is more passive than active and would support the mechanisms of particle ingestion proposed by Palade and Bennett, and would argue against a phagocytic process.

PSL has many special advantages for studies of particle uptake by cells and has been used by Sbarra and Karnofsky in studies of leucocyte metabolism during phagocytosis(12). The high degree of uniformity of size, shape and density make it easily recognizable in the electron microscope. The PSL used in the present experiments had a variation of only $\pm$ .006 μ in particles whose average diameter was 0.264 μ. Total number of PSL particles can be measured easily by calculation from dry weight of sample, by the spray droplet method of Backus and Williams(13) or by sedimentation counting as was done in the above experiments. Finally, PSL can be obtained in sizes ranging from 80 to 1,000 or more millimicrons per lot. These characteristics make feasible a variety of cell-particle experiments of both a qualitative and quantitative nature relating to the general problem of how extracellular material is ingested by cells.

Summary. Ingestion of polystyrene latex (PSL) particles measuring 0.264 μ in diameter has been demonstrated in chicken fibroblasts *in vitro*. PSL was added to cultures of chicken fibroblasts grown on carbon-collodion coated steel grids and these mounts removed at intervals of ½ to 24 hours. The fixed and metal shadowed whole cell preparations revealed PSL in various stages of entry into these cells. PSL was found in invaginations of the cell wall and in vacuoles in the cytoplasm. Sections of cultured fibroblasts inoculated with PSL revealed PSL particles in cell vacuoles. The rate of particle uptake, however, was too low to be measured by the method used.

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Studies Suggesting the Presence of Intrinsic Factor in Bile.* (26789)

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Evidence has previously been presented suggesting the presence of a "circulating intrinsic factor" involved in selective deposition of B_{12} in the liver(1). The present report exhibits 4 lines of evidence suggesting that intrinsic factor may be present in bile.

Materials and methods. Human bile was collected *via* a mushroom catheter in the gall bladder of a patient with complete occlusion of the common duct by a carcinoma of the head of the pancreas. Rat bile was obtained by means of catheterization,† under temporary anesthesia, of the bile duct of rats thereafter maintained for 6 to 24 hours in a special restraining cage with access to food and water.

Hog intrinsic factor concentrate (HIFC), WES 671 A, and purified HIFC, were obtained from the same source.‡ Their activity in man had been demonstrated by a modified Schilling test(2) showing a standard effect in pernicious anemia with single oral doses of 5 mg and of 0.3 mg, respectively. The Co^{60}-B_{12} used was of specific activity approximately 1 $\mu c/\mu g$.§

The liver homogenate system used has previously been described(3); each of the serial incubations was for ½ hour, in air, at room temperature. Everted sacs of rat small intestine were prepared and incubated during procedures carried out essentially as described by others(4). In all experiments a minimum of 10,000 counts were obtained on each sample. Samples which were less than twice background were counted for 10,000 counts on each of 3 separate occasions to ensure validity.

Antiserum to purified HIFC was prepared by injecting into each footpad of a rabbit 0.5 ml of 0.2 mg/ml purified HIFC weekly for a month, and subsequently harvesting serum at intervals separated by further injections of antigen. Agar double diffusion analysis was performed as described by Ouchterlony (5).

Results. Experiment 1, Table I, indicates that human bile increases the enhancing effect of HIFC on Co^{60}-B_{12} uptake by rat liver homogenate in a sequential incubation system. Exp. 2, shows that human bile by itself enhances Co^{60}-B_{12} uptake by rat liver homogenate. Exp. 3, parts a. and b., suggests that the effect of human bile, like that of HIFC(6), is calcium-dependent; part c. demonstrates that the enhancing effect of human bile following incubation is not due to physical entrapment of Co^{60}-B_{12}-laden bile in the liver homogenate. Thus without incubation liver homogenate showed little more uptake from Co^{60}-B_{12}-laden bile than from NaCl-$CaCl_2$.

Table II indicates that, like HIFC, human

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‡ Kindly supplied by Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, N. Y.,

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TABLE I. Enhancement of Frozen, Thawed Liver Homogenate Uptake of Co^{60}-B_{12} by HIFC and by Human Bile.

Exp.	1st incubation Medium	1st incubation Added agents	2nd incubation Medium	2nd incubation Added agent	Counts/min.
1	$NaCl$-$CaCl_2$	NaCl	$NaCl$-$CaCl_2$	Co^{60}-B_{12}	66
	"	HIFC*	"	"	3318
	"	Human bile + HIFC*	"	"	3806
2	KRT,† pH 7.4	NaCl	KRT,† pH 7.4	"	105
	"	HIFC*	"	"	3371
	"	Human bile	"	"	529
	"	Serum from same human	"	"	129
3 (a)	$NaCl$-$CaCl_2$	NaCl	$NaCl$-$CaCl_2$	"	167
	"	HIFC*	"	"	3000
	"	Human bile	"	"	845
(b)	NaCl	NaCl	"	"	263
	"	HIFC*	"	"	302
	"	Human bile	"	"	387
(c)	Human bile + Co^{60}-B_{12} incubated together for 1 hr, without added medium; then added to liver homogenate in $NaCl$-$CaCl_2$, stirred, and homogenate immediately precipitated by centrifugation, washed, then counted				189

* 1/200 daily oral dose of WES671A (25 μg).
† Krebs-Ringer tris, pH 7.4, containing 10 mM $CaCl_2$.

bile will enhance Co^{60}-B_{12} uptake by liver homogenate in a simultaneous incubation system. Furthermore, the HIFC-like action of human bile appears to be calcium-dependent and EDTA-reversible as is the effect of HIFC(6). Table III displays the ability of rabbit antiserum to purified HIFC to eliminate the enhancing effect of both HIFC and human bile. This table also indicates a slight HIFC-like effect of rat bile. Because rat bile showed only this slight intrinsic factor-like activity in the rat liver homogenate system, it was tested in the sensitive *in vitro* everted gut sac system used by Strauss and Wilson(4). Table IV demonstrates that in this system, some samples of rat bile had an intrinsic factor-like activity similar to that of rat stomach homogenate. This was not true for all rat biles tested, however. The concentrations of rat stomach selected for study were based on studies(7) which showed that either much less *or much more* rat stomach homogenate was inactive in the everted sac system.

In Table V, it may be seen that rat bile from each of 2 different donor rats (No. 1

TABLE II. HIFC-like Properties of Human Bile in Rat Liver Homogenate System: Effectiveness in Simultaneous (Bile + Co^{60}-B_{12}) Incubation; Calcium-dependence; EDTA-reversibility.

1st incubation Medium	1st incubation Added agents†	2nd incubation Medium	2nd incubation Added agent†	3rd incubation Medium	3rd incubation Added agent†	Counts/min.
$NaCl$-$CaCl_2$	NaCl + Co^{60}-B_{12}	—	—	—	—	176
"	HIFC* + Co^{60}-B_{12}	—	—	—	—	3178
"	Human bile + Co^{60}-B_{12}	—	—	—	—	573
"	NaCl	$NaCl$-$CaCl_2$	Co^{60}-B_{12}	$NaCl$-$CaCl_2$	—	93
"	HIFC*	"	"	"	—	3748
"	Human bile	"	"	"	—	545
"	NaCl	"	"	"	2 g % EDTA	76
"	HIFC*	"	"	"	"	185
"	Human bile	"	"	"	"	222
NaCl	NaCl	"	"	"	—	86
"	HIFC*	"	"	"	—	103
"	Human bile	"	"	"	—	152

* 25 μg. † 1 ml.

TABLE III. Effect of Rabbit Antiserum to Purified HIFC on Enhancing Action of HIFC and of Human Bile on Co^{60}-B_{12} Uptake by Rat Liver Homogenate. Slight effect of rat bile.

Exp.	1st incubation*	2nd incubation*	3rd incubation*	Counts/min.
a.	NaCl + Co^{60}-B_{12}			132
	Rat bile #1 + Co^{60}-B_{12}			162
	" " #2 + "			191
	HIFC + "			2779
	HIFC† + "			2951
	Human bile + "			318
	(HIFC + rabbit antiserum)† + Co^{60}-B_{12}			51
	(Human bile + rabbit antiserum)† + Co^{60}-B_{12}			80
b.	HIFC	Rabbit antiserum	Co^{60}-B_{12}	674
	Rabbit antiserum	HIFC	"	2081
	HIFC	NaCl	"	3639
	NaCl	HIFC	"	2872

* All incubations were performed in Krebs-Ringer tris (KRT) medium, pH 7.4.
† Preincubated for 1 hr at 37°C before adding to liver homogenate.

and No. 3) appeared to enhance Co^{60}-B_{12} absorption by a gastrectomized rat to the same extent as did the homogenate of ½ rat stomach. However, bile from 2 other donor rats (No. 2 and No. 4) had no such effect.

Ouchterlony agar gel diffusion analysis showed (Fig. 1) a continuous precipitin band (reaction of identity) produced against purified HIFC and rat stomach homogenate by rabbit antiserum against HIFC, with a partial reaction of identity against human stomach homogenate. Similar diffusion analysis utilizing the same antibody demonstrated that a common precipitin line formed with human bile, human stomach homogenate and HIFC.

Discussion. The studies reported here suggest that intrinsic factor is present in bile. Thus, human, and to a lesser extent, rat, bile has HIFC-like activity in the rat liver homogenate semiquantitative "assay" for intrinsic factor. The activity of human bile is calcium-dependent and EDTA-reversible, as is the effect of HIFC. Some samples of rat bile appear to function like rat intrinsic factor in the rat small intestine everted sac qualitative "assay" for rat intrinsic factor. Human bile and intrinsic factor concentrates from 3 species (hog, rat, man) contain a related antigen, demonstrated by agar gel diffusion studies with an antiserum to purified HIFC. Finally, bile from 2 of 4 donor rats appears to possess intrinsic factor activity in a gastrectomized rat.

TABLE IV. Substitution of Rat Bile for Rat Stomach Homogenate in Everted Sacs from Middle of Rat Small Intestine.

Added agent	Counts/min. per 2 inch sac	Enhancement of B_{12} uptake (% above control)
None (control)	23	0
Homogenate of 1/100 rat stomach	113	491
.1 ml rat bile #1	60	261
" " " #2	85	369
" " " #3	44	192

Krebs-Henseleit bicarbonate incubation medium.

TABLE V. Enhancement of Co^{60}-B_{12} Absorption in Gastrectomized Rat Produced by Rat Bile.

Orally administered agents	% of administered Co^{60}-B_{12} in 7-day stool collection
Co^{60}-B_{12}* + 2 ml H_2O	97.4
Idem + ½ rat stomach (homogenate)	77.0
" + 2.2 ml rat #1 bile	76.6
" + 2 ml rat #2 bile	94.3
" + ½ rat stomach (homogenate)	76.1
" + 2 ml rat #3 bile	77.0
" + 2 ml rat #4 bile	95.0
" + 2 ml rat #4 bile	93.8

* 15,000 $\mu\mu$g in 1.5 ml 0.9% NaCl.

While these 4 separate lines of evidence are consistent with the conclusion that intrinsic factor is present in bile, the following equivocations must be introduced: 1. No *in vitro* test can be proved to assay intrinsic factor at the present time, since intrinsic factor has not as yet been isolated in pure form. Thus, both the liver homogenate and everted sac systems may actually measure a contaminant

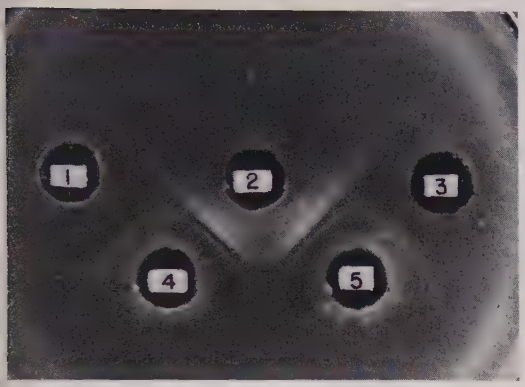

FIG. 1. Ouchterlony plate showing common precipitin line between rabbit anti-hog intrinsic factor and intrinsic factor concentrates from hog, rat, and human sources. 1. Rat stomach homogenate. 2. Purified hog I. F. 3. Human stomach homogenate. 4. Rabbit anti-hog I. F. 5. Rabbit anti-hog I. F.

present in all intrinsic factor concentrates so far prepared, rather than intrinsic factor itself. This possibility cannot be overlooked even though studies so far have shown that besides intrinsic factor only serum(1) and bile produce an enhanced Co^{60}-B_{12} uptake by rat liver systems; other agents either have no effect or many *reduce* the effect of HIFC (8). 2. The present definition of intrinsic factor activity requires that rat bile be repeatedly demonstrated to substitute for rat intrinsic factor in gastrectomized rats before it can be accepted to contain intrinsic factor activity. 3. The immunologic evidence that bile and intrinsic factor concentrates, even from different species, seem to possess a common antigen does not necessarily mean that the common antigen is intrinsic factor.

The results of the semi-quantitative liver homogenate "assay" suggest that amounts of human bile in excess of 1 liter would be necessary to produce an intrinsic factor effect in man, if in fact such an effect could be produced. No enhancement of Co^{60}-B_{12} uptake was obtained in Schilling(2) tests of 2 pernicious anemia patients and of one man who had undergone total gastrectomy when they were fed Co^{60}-B_{12} plus approximately 200 ml of human bile(9). Since this amount of bile is somewhat nauseating, it may be necessary to concentrate the "intrinsic factor activity" of much larger quantities of bile before feeding it or supplying it through a nasogastric tube.

The concept that bile contains intrinsic factor would fit well with the concept of a "circulating intrinsic factor"(1) involved in selective deposition of Vit. B_{12} in liver, and with the concept of the enterohepatic circulation of Vit. B_{12}(11-13) dependent upon or associated with an enterohepatic circulation of intrinsic factor. Toporek found that bile from rat liver which had been perfused with HIFC enhanced Co^{60}-B_{12} uptake in a second perfused rat liver(14), as did HIFC mixed with Co^{60}-B_{12}(15). While this later finding (14) may only mean that a complex of B_{12} bound to non-intrinsic factor B_{12}-binding substance in bile(11) was taken up by Kupffer cells as a foreign material(16), it could mean that intrinsic factor did in fact traverse the hepatic cells, as Toporek suggests. It may be possible to judge between these possibilities, and others, when his study is reported in full.

In observations aimed at narrowing down the possible sources of human intrinsic factor, Castle, Townsend and Heath(10), using a triple-lumen tube during occlusion of the pylorus by a balloon, secured bile-containing duodenal contents. On feeding approximately 75 cc of this material daily for 10 days to a patient with untreated pernicious anemia, they observed a reticulocyte peak of 4.8% (on day 8); whereas in a succeeding period, 75 cc of gastric juice caused a reticulocyte peak of 28.4% (on day 8). The conclusion was drawn that there was no intrinsic factor in bile and that the initial slight hematopoietic effect might have been due to traces of gastric juice entering the duodenum. Now, after a lapse of 3 decades, the possibility that, in the presence of a normally functioning stomach, bile may contain intrinsic factor is again raised. Indeed, intrinsic factor in liver cells or bile might explain the recent finding of Reizenstein and Nyberg(17) that intestinal absorption of liver-bound B_{12} exceeds that of an equivalent amount of B_{12} alone.

The failure of Co^{60}-B_{12} to be absorbed in man(18) and in the rat(19) after total gastrectomy suggests that, if bile does contain

intrinsic factor, its site of origin is the stomach. If absorbed from the intestine after binding Vit. B_{12}, its presence in bile would argue strongly for an entero-hepatic circulation of intrinsic factor in addition to those intestinal phases previously reviewed(20). Consequently, an important question to be answered is whether gastric intrinsic factor is absorbed directly into the blood stream like uropepsin from the cells which make it or is discharged into the stomach and then absorbed with Vit. B_{12} from the ileum after passage into the small intestine.

There are a number of possible explanations for our failure to find intrinsic factor activity in all specimens of rat bile, in studies depending on ability of intrinsic factor to bind radioactive B_{12}. They include: 1) Intrinsic factor may not be secreted at a constant rate in rat bile (too little or too much would be ineffective); 2) The considerable non-radioactive B_{12} content of bile(11-13) may intermittently be great enough to saturate all the intrinsic factor present. (We find an average of 3,000 to 5,000 $\mu\mu$g B_{12} normally present in 1 ml of rat bile by *Euglena gracilis*(21) assay.) Indeed, an unsuspected large isotope dilution effect may explain the apparent inhibition of B_{12} uptake by rat bile reported by others(22,23), whose analyses revealed only 3 $\mu\mu$g B_{12} per ml rat bile; 3) There may not be intrinsic factor in bile.

Summary. Four lines of evidence are presented suggesting that rat and human bile may contain intrinsic factor: 1. Human, and to a lesser extent rat, bile enhances Co^{60}-B_{12} uptake by rat liver homogenate. 2. Some rat bile specimens enhance Co^{60}-B_{12} uptake by everted sacs of rat small intestine. 3. Some rat bile specimens appear to substitute for rat intrinsic factor in a gastrectomized rat. 4. In Ouchterlony agar double diffusion analysis a reaction of identity was formed by human bile and intrinsic factor concentrates from hog, rat, and human sources to antibody induced by injection of purified hog intrinsic factor concentrate. If these findings in fact indicate that bile contains intrinsic factor, it is possible that an enterohepatic circulation of intrinsic factor exists as a further phase of its activity with relation to Vit. B_{12}.

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Suppression of Tubular Secretion of Urate by Pyrazinamide in the Dog.* (26790)

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Pyrazinoic acid and its amide, pyrazinamide (an antituberculous agent), decrease renal excretion of urate in man, causing hyperuricemia(1-3). This is not due to less urate filtered at the glomerulus(3), hence the fall in C_{urate} must be ascribed to modification in the tubules, but whether it is the result of decreased tubular secretion or increased tubular reabsorption of urate has not been determined(3). The present study, using stop-flow and standard clearance procedures, indicates that, at least in the Dalmatian coach hound and apparently also in the non-Dalmatian dog, pyrazinamide suppresses tubular secretion of urate.

Methods. Eight experiments were carried out in female Dalmatian coach hounds, 7 in female mongrel dogs. The dogs weighed 17-21 kg. Anesthesia was not used, except for stop-flow studies (pentobarbital sodium, 30 mg/kg). Standard renal clearance technics were employed, usually under conditions of moderate mannitol or saline diuresis, with appropriate amounts of creatinine and urate added to the infusion. In 7 standard clearance experiments a priming dose of 0.5 g pyrazinamide was delivered rapidly, within 6 minutes, without concomitant mannitol diuresis; in the remaining studies a priming dose of 1.0 or 0.75 g pyrazinamide was delivered over 18 to 27 minutes, and mannitol diuresis was employed. Following the priming dose, a sustaining infusion of pyrazinamide was given at a constant rate, variously 2 to 10 mg/min. Urine and plasma specimens were collected for 56 to 87 minutes after pyrazinamide priming. Each collection period was 10 to 15 minutes in duration.

Three stop-flow studies were carried out, as previously described(4). Control stop-flow collections were first made, then pyrazinamide was infused as described. After 45 to 60 minutes, when a maximum pyrazinamide effect was anticipated, stop-flow urine samples were again collected. The analytical technics employed were as previously described(4).

Results. Dalmatian coach hound. Table I cites a representative standard clearance study, showing a fall in $C_{urate}/C_{creatinine}$ from a mean control ratio of 1.40, indicative of tubular secretion of urate, to a minimum ratio of 0.91 after infusion of pyrazinamide. Table II indicates definite declines in $C_{urate}/C_{creatinine}$ in 5 of 8 such experiments, little or none in the others in which only 0.5 g pyrazinamide was given to prime. C_{urate} fell sharply in the 3 experiments in which a 1.0 g priming dose was used. The glomerular filtration rate, as measured by $C_{creatinine}$, held relatively steady in most experiments; UV_{urate} was variably affected; P_{urate} tended to rise with constant urate infusion rates.

Fig. 1 gives the results of a stop-flow study before and after injection of pyrazinamide in a Dalmatian coach hound infused with urate at a rate of 5 mg/min. In the prestasis free flow periods of the control study, the mean $U/P_{urate}:U/P_{creatinine}$ ratio was 1.34, with urine flow 8.2 ml/min., P_{urate} 3.0 mg% and $U/P_{creatinine}$ 4.11 ml/min. Poststasis, the $U/P_{urate}:U/P_{creatinine}$ ratios also uniformly exceeded unity, with a peak value of 2.0 in the proximal segment (*i.e.*, in urine samples constituting the 80-100% aliquot of the tubular volume and the site of peak PAH secretion); and in the more distal tubular segments the ratios approximated 1.58. In the poststasis free flow periods the $U/P_{urate}:U/P_{creatinine}$ ratios returned to prestasis levels. After injection of pyrazinamide the proximal and distal secretory peaks for urate were almost completely abolished (Fig. 1), the $U/P_{urate}:U/P_{creatinine}$ ratios now approximating unity. Secretion of PAH, in contrast, was comparatively little affected by pyrazinamide.

Mongrels. In the experiments with non-Dalmatian dogs (Table II), net tubular se-

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TABLE I. Representative Experiment Showing Effect of Pyrazinamide on Urate Excretion in Dalmatian (#2147, 16.5 kg, ♀).

Time, min.	V, ml/min.	$C_{creatinine}$, ml/min.	P_{urate}, mg %	UV_{urate}, mg/min.	C_{urate}, ml/min.	$\frac{C_{urate}}{C_{creatinine}}$
0– 5	Infuse creatinine 2.0 g					
5	S_1 started: 0.175% creatinine, 15% mannitol, 0.05% uric acid, 10 ml/min.					
45– 75	15.5	58.0	2.9	2.35	81.0	1.40
75– 96	Pyrazinamide 1.0 g intrav.					
96	S_2 replaces S_1: Pyrazinamide 1.0 g in 1000 ml S_1, 10 ml/min.					
96–105	17.7	49.3	4.0	2.20	54.8	1.11
105–135	22.7	57.8	4.9	2.73	55.6	.96
135–155	22.2	57.6	5.3	2.90	54.8	.95
155–173	19.4	51.8	5.5	2.60	47.2	.91

cretion of urate was not demonstrated ($C_{urate}/C_{creatinine} < 1$) presumably because urate loads and mannitol diuresis were moderate and adjuvant uricosuric agents were not employed (cf. #4). Nevertheless, some decline in $C_{urate}/C_{creatinine}$ occurred in the 5 experiments in which 1.0 or 0.75 g pyrazinamide was given as a priming dose. There was no consistent effect on glomerular filtration rate; changes in UV_{urate} and P_{urate} were variable. In general, the effects of pyrazinamide in the mongrels were similar to those observed in the Dalmatians but the decrease in C_{urate}/GFR was somewhat less pronounced.

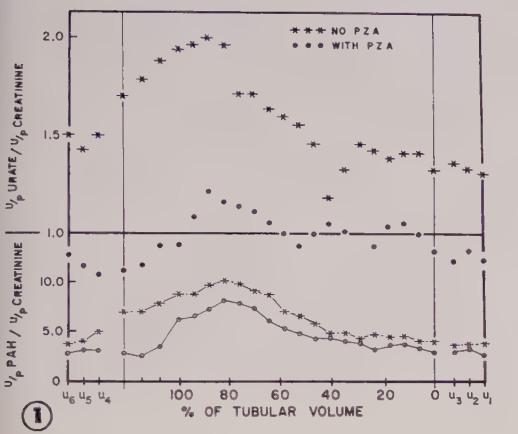
The results of a stop-flow experiment before and after injection of pyrazinamide are shown in Fig. 2. In the control study, $U/P_{urate}:U/P_{creatinine}$ averaged 0.62 in both prestasis and poststasis free flow periods, with urine flow 11.8 ml/min, P_{urate} 8.4 mg%, $U/P_{creatinine}$ 2.99 ml/min. A characteristic dip in the $U/P_{urate}:U/P_{creatinine}$ curve, indicative of net tubular reabsorption of urate, is noted in the proximal tubule. As shown in Fig. 2, this proximal segment of the curve was not altered by pyrazinamide, but there was a discernible decline in $U/P_{urate}:U/P_{creatinine}$ ratios in the more distal portions of the tubule. Little suppressive effect on PAH

TABLE II. Effect of Pyrazinamide on Urate Excretion in Dogs.

Dog	PZA dose: Prime, g	PZA dose: Sustaining, mg/min.	V, ml/min. C	V, ml/min. E	$C_{creatinine}$, ml/min. C	$C_{creatinine}$, ml/min. E	P_{urate}, mg % C	P_{urate}, mg % E	UV_{urate}, mg/min. C	UV_{urate}, mg/min. E	C_{urate}, ml/min. C	C_{urate}, ml/min. E	$\frac{C_{urate}}{C_{creatinine}}$ C	$\frac{C_{urate}}{C_{creatinine}}$ E
Dalmatian														
2147	1.0	10.0	15.5	20.8	58.0	54.7	2.9	5.4	2.35	2.75	81.0	50.9	1.40	.93
2190	1.0	10.0	11.0	20.1	51.5	69.8	3.5	5.2	2.53	3.05	72.3	43.7	1.40	.63
2190	1.0	10.0	20.0	21.6	62.6	71.1	3.1	3.9	2.92	2.37	90.6	60.8	1.45	.86
1834	.5	15.0	32.0	30.1	76.1	70.1	5.8	7.6	5.69	6.54	98.0	86.0	1.29	1.23
"Mat."	.5	5.5	3.7	4.8	81.6	80.4	1.3	1.9	.82	1.06	63.1	55.8	.77	.69
"Mar."	.5	4.0	1.0	1.7	27.8	34.1	3.0	4.0	.85	.92	28.3	23.0	1.02	.68
"Mar."	.5	2.0	.8	.8	25.4	31.0	1.4	1.4	.73	.71	52.2	50.8	2.06	1.63
"Vi."	.5	2.0	3.9	6.6	80.4	84.0	2.2	1.8	2.16	1.96	98.2	109	1.22	1.30
Non-Dalmatian														
2334	1.0	10.0	15.3	22.4	73.4	85.4	1.9	2.0	.86	.87	45.3	43.5	.62	.51
2316	1.0	10.0	13.0	21.1	48.9	56.4	2.6	4.9	1.07	1.54	41.2	31.4	.84	.56
2508*	1.0	8.5	17.1	25.3	81.3	83.3	7.8	9.7	4.54	5.07	58.2	52.3	.72	.63
2453*	1.0	10.0	26.6	22.6	71.0	58.0	8.6	11.4	3.98	3.32	46.3	29.1	.65	.50
2334	.75	9.0	25.1	21.8	90.0	84.8	4.0	3.9	2.83	2.18	70.8	55.9	.79	.66
"Ti."	.5	4.0	1.0	5.0	63.3	76.6	2.3	2.8	.47	.78	20.4	27.9	.32	.36
"Bl."	.5	4.0	.6	2.1	66.4	67.7	4.7	4.0	1.62	1.39	35.5	34.8	.53	.51

C = mean of 3 or 4 control periods. E = mean of last four 10-15 min. experimental periods.

* Urate was infused at 32 mg/min.; in the remaining experiments rate of infusion ranged between 2-10 mg/min.



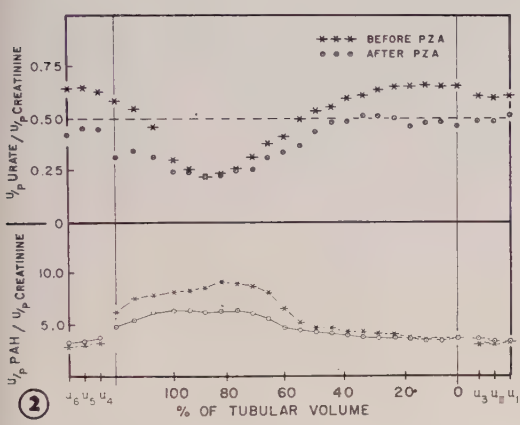


FIG. 1. Stop-flow $U/P_{urate}:U/P_{creatinine}$ ratios (above) in a Dalmatian dog before and after inj. of pyrazinamide. Proximal and more distal secretory urate peaks were almost completely abolished by pyrazinamide. There was little decrease in PAH secretion (lower curves).

FIG. 2. Stop-flow $U/P_{urate}:U/P_{creatinine}$ ratios (above) in a mongrel before and after inj. of pyrazinamide. The proximal trough, indicative of tubular reabsorption of urate, was unaffected by pyrazinamide whereas U/P ratios of the more distal peak were reduced. There was comparatively little decrease in PAH secretion (lower curves).

secretion was again noted.

Discussion. The Dalmatian coach hound is peculiarly suited to investigation of the problem at hand, that of determining whether a decline in C_{urate}/GFR is due to decreased tubular secretion or increased tubular reabsorption of urate. This distinction ordinarily cannot be made in over-all clearance studies but, in the Dalmatian, since tubular reabsorption of urate is defective whereas urate secretion is readily demonstrable(4,5), it seems permissible, because of this dissociation, to attribute lowering of C_{urate}/GFR by a drug to suppression of tubular secretion of urate. It is shown that pyrazinamide in appropriate dosage does, in fact, unequivocally depress C_{urate}/GFR in this species. The inference that this is due to suppression of tubular secretion of urate is fully borne out by the demonstration of abolition of the secretory peaks for urate in stop-flow curves.

It is shown further in these studies that pyrazinamide in appropriate dosage also depresses C_{urate}/GFR in the non-Dalmatian dog, although apparently somewhat less markedly. This effect likewise appears to be due to suppression of tubular secretion of urate. The conditions of the experiments herein cited were not such as to demonstrate net secretion of urate in mongrels (cf. 4). Nevertheless, stop-flow studies reveal no alteration by pyrazinamide in the low U/P_{urate}: $U/P_{creatinine}$ ratios in the proximal tubule, the site of net reabsorption of urate in the mongrel(4,5), but a distinct decline in the higher $U/P_{urate}:U/P_{creatinine}$ ratios in the more distal tubular segments, where net secretion of urate can be demonstrated, under appropriate experimental conditions, in the non-Dalmatian as well as Dalmatian dog (4).

Pyrazinamide causes much less reduction in C_{urate}/GFR in the dog, certainly in the mongrel, than in man(3). The qualitative effects on urate clearance and other discrete renal functions appear to be comparable, however. This applies also to the relative immunity of PAH to pyrazinamide in man (3) and dog, at least when compared to other drugs affecting tubular transport of urate, such as probenecid(6). In the chicken, on the other hand, pyrazinamide has no effect on tubular secretion of urate, except in massive dosage(7). The chicken differs markedly in its response to a variety of other drugs which affect tubular transport of urate in man and dog in a similar manner(7).

The results in the dog may well bear upon the original question(3), whether urate retention caused by pyrazinamide in man is due to decreased tubular secretion or increased tubular reabsorption of urate. At the time these alternatives were suggested,

tubular reabsorption of urate was well known to occur in man(8) whereas evidence for tubular secretion of urate, except perhaps for one abnormal instance(9), was lacking. Such evidence has since been secured, under appropriate experimental conditions, in normal man(10), as well as in the rabbit(11) and dog(4,5,12), suggesting that the mammalian kidney does indeed possess the capacity for tubular secretion of urate. There appears to be at least one precedent for inhibition of tubular secretion of urate in man, the retention of urate caused by salicylate in low dosage, presumably by competition with urate for active tubular transport(13).

Summary. Pyrazinamide depresses C_{urate}/GFR in the Dalmatian and non-Dalmatian dog. Stop-flow studies revealed unequivocal suppression of tubular secretion of urate in the Dalmatian and gave no indication of enhancement of tubular reabsorption of urate in the non-Dalmatian.

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Studies on the Sex Difference in Toxicity of a Cholinergic Phosphorothioate.* (26791)

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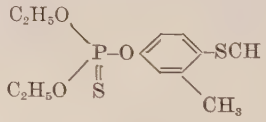
A sex difference in the acute toxicity of parathion to rats was observed in this laboratory in 1949(1). Subsequent studies have established that male rats exhibit greater resistance than females to a number of other phosphorothioates. The mechanism underlying this effect has been difficult to investigate in intact animals because the magnitude of the sex difference in susceptibility is generally relatively small. During measurements of the influence of structural changes on the toxicity of some phosphorothioates we observed a 10-fold sex difference in susceptibility of rats to 0,0-diethyl 0-(4-methylthio-m-tolyl) phosphorothioate (DMP; Bayer 29492). This compound has the following chemical structure:



The magnitude of the sex difference in susceptibility of rats to this phosphorothioate was sufficient to permit a study of factors which might be responsible for the greater resistance of male rats. The present communication describes the mammalian toxicity and anticholinesterase activity of DMP and provides evidence that the liver, under the influence of androgens, is responsible for the resistance of males toward the acute toxicity of this organic phosphate.

* Supported by a grant from U. S. Public Health Service.

TABLE I. Acute Toxicity of DMP to Mammals.

Species	Sex	Route	No. of animals	LD_{50} (mg/kg)
Rats	♀	I.P.	50	22
"	♂	"	52	200
"	♀	Oral	40	14
"	♂	"	40	95
Mice	♀	I.P.	35	25
"	♂	"	40	35
Guinea pigs	♂	"	32	30

Materials and methods. Male and female Sprague-Dawley rats weighing between 175 and 250 g were used unless otherwise specified. Young male guinea pigs (*ca* 200 g) and Carworth Farms mice (*ca* 20 g) were used. The organic phosphates, which were kindly supplied by the Chemagro Corp., Kansas City, Mo., were dissolved in a mixture of 20% ethanol and 80% propylene glycol and concentration of toxic agent was so adjusted that animals always received amounts of solution equivalent to less than 1% of their body weight. Testosterone propionate was dissolved in sesame oil (2 mg/ml) and administered subcutaneously. Cholinesterase assays were performed manometrically by the method of DuBois and Mangun(2). In the toxicity measurements animals were observed for 10 days and LD_{50} values were calculated from the mortality data by the logarithm-probability method.

Results. Table I shows the acute toxicity of DMP to rats, mice and guinea pigs. These results indicated that this compound has a relatively high and similar toxicity to mammals with the exception of male rats which exhibited a marked resistance. The 10-fold difference in susceptibility of male and female rats to DMP given intraperitoneally greatly exceeds the magnitude of the sex differences noted previously with other phosphorothioates. It was sufficiently great to permit further toxicity experiments to clarify the factors responsible for this effect.

The symptoms produced by DMP were typical of those caused by cholinergic phosphorothioates. Following administration of the compound to rats the cholinesterase activity of peripheral tissues and brain was markedly inhibited. Fig. 1 shows the inhibitory action of a sublethal dose (125 mg/kg) of DMP on cholinesterase activity of brain, submaxillary glands and serum of male rats and rate of reversal of the inhibition. Each point on the curves represents an average for tissues from 3 animals. Maximum inhibition occurred within 6 hours and persisted for about 24 hours after which gradual reversal occurred. Similar measurements performed on female rats given 13.75 mg/kg, which is the same fraction of the LD_{50} used for the assays on males, demonstrated that equitoxic doses produce the same amount of inhibition of cholinesterase and the same rate of recovery in males and females.

Phosphorothioates do not have anticholinesterase activity but they are converted to toxic metabolites *in vivo* by the liver of mammals through replacement of the sulfur of the thiophosphate linkage by oxygen(3,4,5). The resulting oxygen analogues are, therefore, directly responsible for the toxicity and anticholinesterase activity. Measurements of the toxicity of the oxygen analogue of DMP (0,0-diethyl 0-[4-methylthio-m-tolyl] phosphate) to rats (Table II) demonstrated that there is no sex difference in rats to this highly toxic compound, suggesting that the sex difference in acute toxicity of the parent compound may be due either to a difference in rate of conversion of the sulfur to the oxygen analogue or rate of detoxification of the sulfur analogue.

In a search for information on factors involved in the sex difference in toxicity of

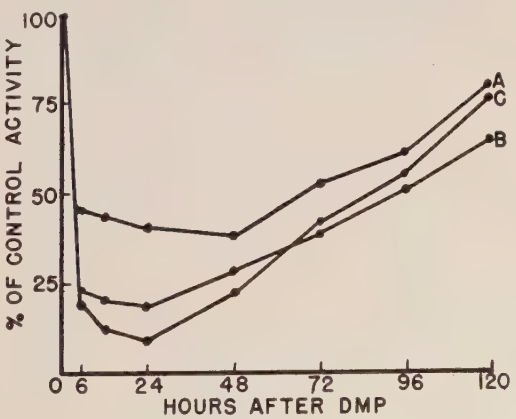


FIG. 1. Rate of decrease and recovery of cholinesterase activity of tissues from male rats after intraper. inj. of DMP (125 mg/kg). A. Brain. B. Submaxillary. C. Serum.

TABLE II. Acute Toxicity of the Oxygen Analogue of DMP to Rats.

Sex	Route	No. of animals	LD_{50} (mg/kg)
♀	I.P.	35	5.7
♂	"	30	5.8
♀	Oral	35	9.0
♂	"	40	12.0

DMP the possibility was considered that the liver, under some regulatory influence of androgens, was responsible. A number of toxicity tests were, therefore, conducted on male rats subjected to partial hepatectomy, castration, and treatment with androgens. The results are summarized in Table III. At 2 days after removal of the lateral and medial lobes of the liver the resistance of male rats to DMP was absent as indicated by a decrease in LD_{50} from 200 to 16 mg/kg. When the liver was allowed to regenerate for 14 days, the animals reacquired most of their resistance. However, when males were castrated, subjected to partial hepatectomy one week later and treated with DMP at 14 days following hepatectomy they failed to regain their resistance. This finding suggested that development of the system in the liver which accounts for the resistance of male rats to DMP is under the influence of androgens. This possibility received further support by repetition of this experiment with addition of daily subcutaneous injections of testosterone (2 mg/kg/day) from time of hepatectomy until DMP was given. The approximate LD_{50} of DMP to these animals was 150 mg/kg. Other evidence for the possible role of androgens as a factor involved in the resistance of males was obtained by measuring

TABLE III. Influence of Partial Hepatectomy, Castration and Testosterone Treatment on Acute Toxicity of DMP to Male Rats.

Treatment	No. of animals	I.P. LD_{50} (mg/kg)
None	50	200
2 days after partial hepatectomy	35	16
14 days after partial hepatectomy	32	160
14 days after partial hepatectomy and castration	24	25
Idem, and testosterone (2 mg/kg/day)	31	140
14 days after castration	32	175

the toxicity of DMP to young male rats. The LD_{50} for 23-day-old males was 15 mg/kg and at 45 days of age the value was 210 mg/kg. The resistance was, therefore, acquired within the period of puberty in male rats(6).

Additional experiments were performed on adult female rats to ascertain whether their high susceptibility could be altered by treatment with testosterone. For these measurements normal and ovariectomized female rats were given 2 mg/kg of testosterone propionate for a period of 30 days. The LD_{50} of DMP to these animals increased from 25 to about 100 mg/kg. Ovariectomy itself had no effect on the susceptibility. At 24 hours after a single dose of testosterone propionate (2 mg/kg) no effect was noted on the susceptibility of female rats to this toxic agent indicating that testosterone does not exert a direct activating effect on the system responsible for the resistance.

Discussion. The toxicity of phosphorothioates is governed by 3 biochemical reactions, namely (a) rate and extent of metabolic conversion in the liver to their oxygen analogues, (b) potency of the oxygen analogues as inhibitors of cholinesterase and (c) rate and extent of detoxification of parent compounds and oxygen analogues. Our finding that there is no sex difference in the toxicity of the oxygen analogue of DMP to rats eliminated anticholinesterase action and detoxification of the oxygen analogue from further consideration in this study. It, therefore, seemed likely that either the rate of metabolic conversion of DMP to its oxygen analogue or detoxification of the parent compound was responsible for the sex difference in susceptibility.

Previous studies in this laboratory(7) have shown that the concentration of the enzyme which catalyzes the desulfuration of phosphorothioates is higher in livers of males than of female rats. On this basis males would be expected to be more susceptible than females unless the rate of metabolic conversion to the toxic oxygen analogue by the livers of male rats is inhibited *in vivo* by some factor which is absent from *in vitro* liver systems. The biochemical reactions involved in detoxification of this phosphorothioate

have not been investigated. It, therefore, remains to determine the exact biochemical mechanism responsible for the sex difference in toxicity of DMP and other phosphorothioates. However, through the use of partially hepatectomized male rats the liver was found to be the organ directly responsible for the sex difference in susceptibility. Experiments with partially hepatectomized, castrated and testosterone-treated male rats, as well as weanling males, provided information suggesting that synthesis of some component of a system in liver which affects the toxicity of phosphorothioates to male rats is stimulated by testosterone. These findings may, therefore, serve as a basis for further studies aimed at identification of the exact biochemical process responsible for the sex difference in susceptibility of rats to phosphorothioates.

Summary. Measurements of the acute toxicity of DMP, a new cholinergic phosphorothioate, demonstrated that it has a high toxicity to female rats, male and female mice and male guinea pigs as evidenced by intraperitoneal LD_{50} values ranging from 22 to 35 mg/kg. An exception was noted in the case of male rats to which the intraperitoneal LD_{50} was 200 mg/kg. DMP inhibits cholinesterase activity of brain and peripheral tissues *in vivo*. The LD_{50} of the oxygen analogue of DMP was 5.8 mg/kg to male rats and there was no sex difference in susceptibility. The 10-fold resistance of male rats to the toxicity of DMP was absent at 2 days after partial hepatectomy. At 14 days after partial hepatectomy males regained their resistance unless they were castrated before hepatectomy. Injection of testosterone caused redevelopment of resistance in hepatectomized, castrated rats. The results of these experiments indicate that some system in the liver is responsible for the resistance of male rats to DMP and that androgens govern the development of this system.

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Coenzyme Q Metabolism in Pantothenic Acid Deficiency.* (26792)

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Though the origin of the substituted benzoquinone ring of the coenzyme Q molecule still remains uncertain, the mechanism of biosynthesis of the isoprenoid side chain appears to be well established. Incorporation of intraperitoneally administered labeled mevalonic acid into rat liver coenzyme Q has been reported by Gloor and Wiss(1,2); Dialameh and Olson(3) have since observed that acetate-C^{14} also serves as a precursor of the isoprenoid side chain, *in vivo,* in the rat. Biosynthesis of the side chain of coenzyme Q from these 2 labeled precursors, both *in vivo* (4-6) and *in vitro*(6,7) have been confirmed by others. The well known involvement of coenzyme A in biosynthesis of isoprene units and of mevalonic acid from 2 carbon fragments, suggested a study of the relation of coenzyme Q metabolism to pantothenic acid status of the animal. Observations on endogenous tissue levels of coenzyme Q as well as on its biosynthesis *in vivo* and *in vitro* from labeled precursors in pantothenic acid de-

* Preliminary report has been published(18).

TABLE I. Intracellular Distribution of Coenzyme Q.

Group*	Pantothenic acid, μg/g	Coenzyme Q, μg/g — Whole liver	Nuclei	Mitochondria	Microsomes	Supernatant	Succinoxidase activity, μl O_2/hr/g
P-supplemented	133.7 ± 6.1	127 ± 7.6	37 ± 4.1	48 ± 2.7	19 ± 3.1	7 ± 1.6	1284 ± 69
P-deficient	79.6 ±11.2	103 ± 3.1	33 ± 5.6	36 ± 4.1	17 ± 4.1	6 ± 2.4	963 ± 74

Results are avg of 4 independent observations ± stand. error of mean, and are expressed on fresh weight basis.

* P = Pantothenic acid.

ficiency are presented and discussed in the light of other reported observations.

Experimental. Induction of deficiency. Deficiency of pantothenic acid was induced by feeding weanling male rats (Wistar strain) a purified casein ration, devoid of the vitamin, for 8 weeks (for details, cf. 18). The control group received a supplement of 20 mg calcium pantothenate, per kg of diet, throughout the experimental period. To one batch of rats fed the deficient diet for 8 weeks, calcium pantothenate (10 mg/rat) was administered intraperitoneally, and the animals were sacrificed at intervals of 0, 8, 24 and 48 hours from time of administration.

Determinations. The livers were quickly excised, chilled in isotonic sucrose (0.25 M) and made into 10% homogenates in the same, using a Potter-Elvehjem type homogeniser. Separation of the homogenate into the subcellular fratcions was carried out by the procedures of Schneider and Hogeboom(8) and of Palade and Siekevitz(9). The nuclei and mitochondria were separated at 700 × g and at 5000 × g for 10 minutes each, in a PR-2 International refrigerated centrifuge, and the microsomes were sedimented at 105,000 × g for 60 minutes in a Spinco model L preparative ultracentrifuge. The supernatant fraction was further freeze-dried. Total pantothenic acid content(10) and succinoxidase activity(11) of liver were assayed essentially as detailed by the respective authors, while coenzyme Q in the unsaponifiable liver lipids was chromatographically purified by the method of Festenstein *et al.*(12) with minor modifications. Absorption of the oxidised and reduced forms (after addition of potassium borohydride) at 272 mμ was read on a Beckman model DU Spectrophotometer(13).

Studies on in vivo *and* in vitro *incorporation of labeled precursors.* Four animals from each group were fasted overnight, intraperitoneally administered either 0.5 ml acetate *U*-C^{14} (20 μc) or mevalonate-2-C^{14} (10 μc), and were sacrificed 4 hours later. The livers were removed, and coenzyme Q was isolated from the unsaponifiable lipids by chromatography on alumina. The fractions containing the coenzyme Q were rechromatographed and the radioactivity of 2 samples pooled together was counted at infinite thinness on stainless steel planchets employing the Tracerlab SC-16 windowless flow gas counter in conjunction with Tracerlab SC-51 autoscaler. After counting, the coenzyme Q in the planchets was determined spectrophotometrically.

For studies on *in vitro* incorporation, liver slices, prepared using a Stadie tissue slicer (A. H. Thomas Co., U.S.A.), and weighing 500 mg from animals of both groups were incubated in Warburg flasks open to atmosphere and containing 10 μM mevalonate-2-C^{14} (10 μc) along with 3 μM disodium salt of adenosine triphosphate, and 60 μM glucose in 4.0 ml of Krebs-Ringer phosphate buffer, pH 7.2. At the end of incubation period, the

TABLE II. Effect of Administration of Pantothenic Acid to Deficient Rats.

Hr after administration	Coenzyme Q, μg/g — Whole liver	Mitochondria	Succinoxidase activity, μl O_2/hr/g
0	103 ± 3.1	36 ± 4.1	963 ± 74
8	111 ± 4.7	40 ± 3.6	1048 ± 42
24	124 ± 6.3	47 ± 1.4	1213 ± 36
48	129 ± 3.1	45 ± .9	1246 ± 98

Calcium pantothenate (10 mg) was inj. intraper. to deficient rats and animals sacrificed 0, 8, 24 and 48 hr later.

Results are avg of 4 independent determinations ± stand. error of mean and are expressed on fresh liver weight.

TABLE III. Incorporation of Acetate-C^{14} and Mevalonate-2-C^{14} into Rat Liver Coenzyme Q *In Vivo*.

Group*	Incorporation into coenzyme Q from Acetate-C^{14} Total counts, cpm	Acetate-C^{14} Specific activity, cpm/mg	Mevalonate-2-C^{14} Total counts, cpm	Mevalonate-2-C^{14} Specific activity, cpm/mg
P-deficient	144 ± 42	831 ± 62	654 ± 35	3316 ± 269
P-supplemented	336 ± 71	1864 ± 221	957 ± 104	4456 ± 492

Four animals from each group were fased overnight and administered intraper. either acetate-*U*-C^{14} (20 μc) or mevalonate-2-C^{14} (10 μc) and were sacrificed 4 hr later.

Results are avg of 4 independent determinations ± stand. error of mean.

* P = Pantothenic acid.

suspending medium was decanted off, the slices washed twice with Krebs-Ringer phosphate buffer, and radioactivity and spectrophotometric determinations of coenzyme Q in pooled slices carried out as described earlier.

Results and discussion. The changes in liver stores of coenzyme Q and its intracellular distribution in pantothenic acid deficiency are presented in Table I. A decrease in level of coenzyme Q, particularly marked in the mitochondrial fraction, is paralleled by reduction in succinoxidase activity. This parallelism between mitochondrial coenzyme Q and succinoxidase activity would be consistent with the reported participation of coenzyme Q in the succinoxidase system(14-17).

Administration of a single dose of calcium pantothenate to deficient animals restores the coenzyme Q levels, with concomitant increase in succinoxidase activity, to almost normal by the end of 24 hours (Table II).

That the decrease in liver stores of coenzyme Q in pantothenic acid deficiency may be due to decreased synthesis, because of the involvement of coenzyme A in biosynthesis of the isoprenoid side chain, appears to be fully borne out by studies on incorporation of labeled precursors into coenzyme Q, both *in vivo* and *in vitro*. The deficient animals show lesser incorporation of the label into coenzyme Q, more particularly seen with acetate (Table III). *In vitro* incorporation of mevalonate-C^{14} into coenzyme Q is far less than *in vivo*, and is possibly due to the lack of certain essential cofactors in the *in vitro* system, as has also been suggested by Phillips(7) (Table IV).

TABLE IV. Incorporation of Mevalonate-2-C^{14} into Rat Liver Coenzyme Q *In Vitro*.

Group*	Incorporation into coenzyme Q Total counts, cpm	Specific activity, cpm/mg
P-deficient	316 ± 24	814 ± 47
P-supplemented	467 ± 29	1162 ± 113

Liver slices (500 mg) were incubated in a system containing 10 μM mevalonate-2-C^{14} (10 μc) along with 3 μM disodium salt of adenosine triphosphate, and 60 μM glucose in 4.0 ml Krebs-Ringer phosphate buffer, pH 7.2 for 3 hr.

Results are avg of 4 independent determinations ± stand. error of mean.

* P = Pantothenic acid.

Summary. 1. Pantothenic acid deficiency results in decreases in coenzyme Q and succinoxidase activity of liver. Administration of pantothenic acid to the deficient animals results in a restoration to normal by the end of 24 hours. 2. The deficient animals show decreased incorporation of labeled acetate and mevalonate into liver coenzyme Q, indicating a decreased synthesis of the coenzyme Q as the possible cause of lowered liver levels of coenzyme Q in pantothenic acid deficiency.

Grateful acknowledgement is made to Dr. O. Wiss, Hoffmann la Roche Ltd., Basle, Switzerland for the kind gift of mevalonic acid-2-C^{14} used in these studies. Our thanks are also due to the Indian Council of Medical Research for financial support and to Dr. V. Subrahmanyan, Director of this Institute, for his interest and encouragement.

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Effect of Vanadium Administration on Coenzyme Q Metabolism in Rats. (26793)

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During studies on the *in vitro* biosynthesis of cholesterol, Curran(1) observed a marked inhibitory effect of vanadium ions on incorporation of acetate-C^{14} into cholesterol by liver homogenates. Similar observations on *in vivo* biosynthesis of cholesterol and of phospholipids in vanadium fed animals have been reported by others(2-4).

While Azarnoff and Curran(5) indicated that the inhibition occurred between the stages of mevalonic acid and β-methyl crotonate, Wright *et al.*(6) suggested that vanadium probably interfered with maintenance of adenosine triphosphate levels in the system and that this may impair the synthesis of phosphorylated intermediates from mevalonic acid. This view is further substantiated by the report that exogenous adenosine triphosphate enhances the synthesis of cholesterol from mevalonic acid(7) and that inhibitors of oxidative phosphorylation decrease it(8). A similar inhibition of acetate incorporation into phospholipids *in vitro* by fluoride, cyanide, dinitrophenol and iodoacetate has been shown earlier by Kline and De Luca(9).

That the decreased synthesis of cholesterol may be due to a lowering in tissue stores of coenzyme A is suggested by the recent work of Mascitelli-Coriandoli and Citterio(10), who have observed considerable reductions in hepatic coenzyme A in rats administered vanadium either orally or parenterally. Other workers(4,11) have suggested that the metabolism of sulfur amino acids may be interfered with by vanadium, in which case a shortage might be postulated of the sulfhydryl reserves of the cell, notably of cysteine, that are needed for biosynthesis of coenzyme A.

The well known involvement of coenzyme A in biosynthesis of isoprene units and of mevalonic acid from 2 carbon fragments and the reported incorporation(12-15) of these into the isoprenoid side chain of coenzyme Q prompted a study of the effect of vanadium administration on coenzyme Q metabolism.

Experimental. Male weanling rats (Wistar strain) 40-50 g in weight were fed a purified 10% casein diet replete in all respects (cf. 16) for a period of 8 weeks. To one group of rats, ammonium vanadate (0.6 mg/rat/week) was administered intraperitoneally while control rats received an equal amount of distilled water. At the end of 8 weeks, some animals from the vanadium ad-

TABLE I. Effect of Vanadium on Growth, Liver Content of Coenzymes A and Q and Succinoxidase Activity.

Group	Growth, g/8 wk	Coenzyme A, units/g	Coenzyme Q, μg/g	Succinoxidase activity, μl O_2/hr/g
10% casein diet	151.8 ± 12.9	164 ± 11	127 ± 7.6	1304 ± 32
Idem + intraper. admin. vanadium	106.4 ± 7.3	89 ± 17	97 ± 9.9	996 ± 51

Results are avg of 6 independent determinations ± stand. error of mean and are on fresh weight basis.

TABLE II. Intracellular Distribution of Coenzyme Q in Vanadium Administered Rat Liver.

Group	Coenzyme Q, μg/g				
	Whole liver	Nuclei	Mitochondria	Microsomes	Supernatant
10% casein diet	127 ± 7.6	37 ± 4.1	48 ± 2.7	19 ± 3.1	7 ± 1.6
Idem + intraper. admin. vanadium	97 ± 9.9	33 ± 4.3	33 ± 1.9	13 ± 1.8	6 ± .9

Results are avg of 6 independent determinations ± stand. error of mean.

ministered group were given calcium pantothenate (10 mg/rat) or l-cysteine hydrochloride (10 mg/rat) or disodium salt of adenosine triphosphate (10 mg/rat) intraperitoneally. A few rats were given all 3 simultaneously. The animals were sacrificed 24 hours after administration of these compounds by decapitation and the livers chilled, homogenised in isotonic sucrose and fractionated according to the procedures of Schneider and Hogeboom(17) and of Palade and Siekevitz(18).

Coenzyme A, coenzyme Q and succinoxidase activity of liver were determined as described earlier(16).

Results and discussion. Vanadium administration results in a lowering of liver stores of coenzyme A (CoA), coenzyme Q (CoQ) and succinoxidase (S.O. activity (Table I). The intracellular distribution of coenzyme Q shows a marked reduction in the mitochondrial fraction with no significant changes in the other fractions (Table II). These changes are similar to those obtained in pantothenic acid deficiency(16,19) and are suggestive of a primary interference by vanadium in the metabolism of either pantothenic acid or its functional form, coenzyme A.

Since a primary lowering in coenzyme A level may lead to decrease in the coenzyme Q stores in liver, the protective effects of administering calcium pantothenate, l-cysteine hydrochloride and disodium salt of adenosine triphosphate either individually or together on liver levels of coenzymes A and Q were studied. The results are presented in Table III. No significant restorative effect was observable with calcium pantothenate or ATP alone, but cysteine was partially effective. The 3 together almost completely reversed the changes due to vanadium administration. The results point to an interference by vanadium in biosynthesis of coenzyme A, possibly through its effect on tissue sulfhydryl(4) and

TABLE III. Protective Effects of Pantothenic Acid, Cysteine and Adenosine Triphosphate against Vanadium Treatment.

Compounds administered			Coenzyme A, units/g	Coenzyme Q, μg/g	Succinoxidase activity, μl O_2/hr/g
Ca-P	CSH	ATP			
+	—	—	98 ± 13	98 ± 8.4	984 ± 52
—	+	—	123 ± 9	109 ± 7.1	1144 ± 36
—	—	+	111 ± 7	106 ± 4.5	1086 ± 49
+	+	+	143 ± 12	123 ± 6.7	1361 ± 71

Calcium pantothenate (Ca-P) (10 mg/rat), l-cysteine hydrochloride (CSH) (10 mg/rat) and disodium salt of adenosine triphosphate (ATP) (10 mg/rat) were given intraper. and the animals sacrificed 24 hr later.

Results are avg of 4 independent determinations ± stand. error of mean and are expressed on fresh weight basis.

adenosine triphosphate(6) reserves.

Summary. Vanadium administration results in decreases in coenzyme A, coenzyme Q and succinoxidase activity in rat livers. A marked protection against these changes is afforded by simultaneous administration of calcium pantothenate, l-cysteine hydrochloride and disodium adenosine triphosphate. The results are discussed in relation to other reported observations on the effects of vanadium administration.

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Cytochrome Oxidase in Radiosensitive and Radioresistant Amoebae.* (26794)

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Two species of the giant amoeba *Pelomyxa* are known to differ by a factor of approximately 10 in their sensitivity to x-radiation. The x-ray LD_{50} for *P. carolinensis* is about 100 kr(1-3), whereas that for *P. illinoisensis* is about 10 kr(2,4,5). It seemed possible that there might be some fundamental metabolic difference responsible for this wide dissimilarity in radiosensitivity. Very little is known about the metabolic characteristics of *P. illinoisensis*. However, *P. carolinensis* is known to contain cytochrome pigments(6), and 70% of its respiration is cyanide-sensitive(7). Møller and Prescott(6) were unable to demonstrate absorption peaks characteristic for cytochrome *c* by direct spectrophotometric examination of ruptured cells, and several investigators have failed to detect cytochrome *c* oxidase activity (see 6). Møller and Prescott suggested that a cytochrome *e*-cytochrome *c* oxidase system was operating as the terminal oxidase in *P. carolinensis*.

It occurred to us that *P. illinoisensis* might contain cytochrome *c* oxidase, in contrast to the more radioresistant species. Thus we assayed the 2 species for this enzyme. We have found, however, that both species contained approximately the same amount of cytochrome *c* oxidase activity, although the sys-

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tem is rather labile, particularly in *P. carolinensis*. For comparative purposes we looked for this enzyme in *Amoeba proteus*. This large uninucleate amoeba is radioresistant, with an LD_{50} of at least 120 kr(2,3,8).

Methods. The *Pelomyxa* species (Illinois strains, isolated by Kudo(9,10)) were grown on *Paramecium multimicronucleatum* and *Chilomonas paramecium*. *Amoeba proteus*, originally obtained from General Biological Supply House, Inc., Chicago (1957), was grown predominantly on *Tetrahymena pyriformis* (strain W). *Paramecium* and *Chilomonas* organisms similar to those which were fed to the pelomyxae were used for supplemental food. All amoebae used in these experiments were starved for 4 days prior to use; they were thoroughly washed before and at daily intervals during starvation to eliminate food remnants.

The amoebae were disrupted by homogenization in ice-cold water. Homogenizers were made from barrels and matched plungers of 0.5 ml syringes, with all surfaces ground to fit closely. The packed volume of cells (one *g* of force for 15 minutes) used in each homogenate was about 0.15 ml for the pelomyxae and 0.06 ml for *Amoeba proteus*, representing approximately 1400 organisms of *P. carolinensis*, 3300 of *P. illinoisensis*, and 16,000 of *A. proteus*. Total volume of each homogenate was about 0.3 ml. Microscopic examinations of homogenates revealed that cellular disruption was complete after 20 or 30 seconds of rotation and compression of the plunger.

Cytochrome oxidase was measured by the spectrophotometric method of Hogeboom and Schneider(11). Aliquots of the homogenate were added to the reaction mixture (reduced cytochrome *c* (Sigma), Al^{+++}, and phosphate buffer, pH 7.4) within one minute after homogenization. Readings were made at intervals of 30 or 60 seconds at 550 mμ to measure the rate of oxidation of reduced cytochrome *c* at 25.0°C. Initial rates were calculated by extrapolation to zero time. Total nitrogen was measured by nesslerization of an H_2SO_4-H_2O_2 digest of a portion of the homogenate.

In some experiments, the homogenates were incubated for varying lengths of time, either at 0°C or 25°C, prior to assay, so that information could be obtained about the stability of the system.

TABLE I. Cytochrome Oxidase Activities of Protozoa and Mammalian Tissues.

Source	No. exp.	μmoles cytochrome *c* oxidized/hr/mg N*
Pelomyxa illinoisensis	3	7.4 ± .1
Pelomyxa carolinensis	3	8.3 ± 1.5
Amoeba proteus	2	.6 ± .3
Paramecium aurelia	3	1.5 ± .7
Rat thymus	9	24.6 ± 1.8
Mouse liver	17	322 ±16

* Averages with avg deviation.

Results. Table I shows the cytochrome *c* oxidase activities of 3 species of amoebae assayed immediately after disruption of the cells. For purposes of comparison, the activities of *Paramecium aurelia* and of 2 mammalian tissues, assayed by the same procedure, are also included. It is clear that the activities of the 2 *Pelomyxa* species are nearly the same, and considerably lower than those of mammalian tissues: ⅓ that of thymus and 1/40 that of liver. Mammalian tissues, especially the thymus, are considerably more radiosensitive than the most radiosensitive of the amoebae that have been studied. *Amoeba proteus* contains an almost negligible amount of cytochrome oxidase; *Paramecium aurelia* has a somewhat higher concentration, but levels of activity of both are extremely low and variable, and at least in the case of *Amoeba proteus* of questionable significance.

The necessity of carrying out the assay as soon as possible after disruption of the cells is revealed by Fig. 1. The activity of the homogenized cells is apparently stable in 0°C, at least for 20 to 40 minutes, but disappears rapidly at 25°C. The activity of *P. illinoisensis* decreased to 50% after 15 minutes, whereas that of *P. carolinensis* dropped to 50% in about 3 minutes. After 45 minutes at room temperature, 80 to 90% of the activity of both species had disappeared. In one experiment in which *P. illinoisensis* was homogenized at room temperature, then assayed about 5 minutes later, the initial rate was only 25% of that observed when homogenization was carried out at 0°C and the sam-

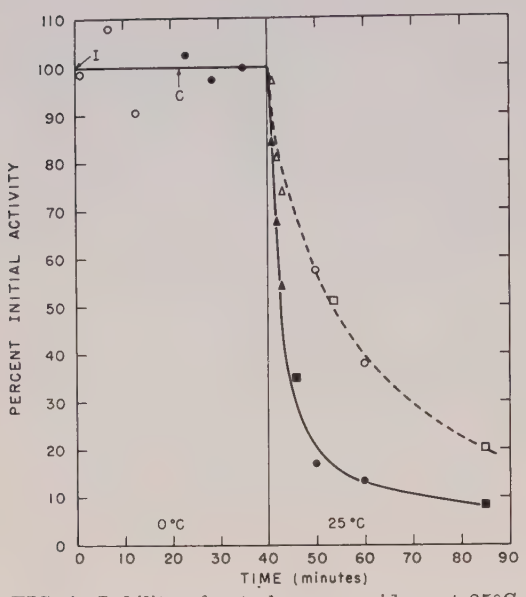

FIG. 1. Lability of cytochrome *c* oxidase at 25°C. Solid line and symbols, *P. carolinensis*; dotted line and open symbols, *P. illinoisensis*. Samples of *P. illinoisensis* were homogenized at 0°C at zero time (arrow at I), and 3 assays were made beginning 1, 7, and 12.5 min. later. *P. carolinensis* was homogenized at 23 min. (arrow at C) and 3 assays similarly made. At 40 min., both homogenates were transferred to a 25°C bath. Readings were made 10 and 20 min. later (circles). Data from other experiments are incorporated in this graph (squares). The points at 41, 42, and 43 min. (triangles) were calculated from rate of decrease of activity during the assays, corrected for anticipated first-order decrease due to substrate utilization.

ple assayed immediately afterward.

To rule out the possibility that the changes observed in optical density represented merely a swelling of subcellular particulates and not a true oxidation of cytochrome *c*, assays were carried out in the presence of 0.006 M NaCN. Virtually complete inhibition occurred; there was negligible change in optical density.

Discussion. It should be emphasized that these experiments indicate only that there is present within the cells of the giant amoebae an enzyme that can catalyze the oxidation of reduced cytochrome *c* by molecular oxygen. The activity of this enzyme is relatively low, and there is no reason to presume that a cytochrome *c*-cytochrome *c* oxidase system represents a significant metabolic pathway *in vivo*, particularly in view of the inability of Møller and Prescott(6) to demonstrate absorption bands of cytochrome *c* in *P. carolinensis*. It is very possible that the protozoan enzyme that oxidizes cytochrome *c in vitro* is not identical with the mammalian enzyme, and that mammalian cytochrome *c* is not its physiological substrate.

According to a recent report(12), cytochrome oxidase (prepared from beef heart) is a copper-hemoprotein complex consisting of equimolar quantities of copper and cytochrome *a*. Møller and Prescott(6) detected an absorption band at 605 mμ, which they attributed to either cytochrome *a*, cytochrome *e* oxidase, or both. They made no mention, however, of the other absorption bands characteristic of cytochrome *a*. Furthermore, the band at 605 mμ was weaker in *P. carolinensis* than in *Amoeba proteus*, whereas according to our data, the latter has much less cytochrome *c* oxidase activity.

Nevertheless, there is now definite evidence that these cells possess some sort of cytochrome *c* oxidase. The extreme lability of the system, particularly in *P. carolinensis*, is probably the reason why this enzyme has not been hitherto detected in these protozoa. Møller and Prescott(6) indicated the necessity of maintaining their homogenates at 0° to −10°C in order to preserve the absorption bands.

Summary. Cytochrome *c* oxidase activity has been demonstrated in 2 species of the giant amoeba *Pelomyxa*, one of which is radioresistant and the other radiosensitive. The differences observed in this enzyme do not appear to be sufficient to account for the 10-fold difference in radiosensitivity of the living organisms. The level of cytochrome *c* oxidase activity is about one-fortieth that of mammalian liver, and is highly labile at room temperature. The radioresistant *Amoeba proteus* was also assayed for cytochrome *c* oxidase activity but almost negligible amounts were found.

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Hypergammaglobulinemia in Mink.* (26795)

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Lesions found in mink affected with Aleutian disease resemble many of those described for such diseases as periarteritis nodosa, lupus erythematosus, and other associated diseases of man. Certain of the gross and microscopic lesions of Aleutian disease have been described by Hartsough and Gorham (1), and Helmboldt and Jungherr(2). Obel (3), in describing a similar condition of mink in Sweden, has pointed out that this may be a form of plasma cell myeloma, since one of the prominent changes is plasma cell proliferation. Recently Page(4) found that affected mink have an accompanying hypergammaglobulinemia. This report is concerned with the determination and comparison of serum protein values of normal and Aleutian disease affected mink using zone electrophoresis.

Materials and methods. Blood samples were taken from normal and affected mink on 2 ranches which had a history of the disease. In addition, blood samples were collected from a ranch where the disease had never occurred and the animals were in good health. The blood was collected from 18 normal and 18 affected mink by heart puncture while the animals were under ether anesthesia. Immediately after collection of the blood samples, the mink were sacrificed and complete necropsies performed. Animals were designated normal or affected on the basis of history, clinical signs, and post-morten lesions. Serum was removed from blood samples and stored at –26°C until electrophoretic determinations were made.

All the mink in the affected group were homozygous for the Aleutian gene as defined by Shackelford(5). The control mink, which had no evidence of disease, included 11 animals which were homozygous for the Aleutian gene and 7 which were not homozygous for this gene.

Samples of serum from normal and affected mink were subjected to electrophoresis at room temperature on filter paper (S and S 2043-A mgl) in veronal buffer (ionic strength 0.075 and pH 8.6) for 16 hours at a constant current of 0.104 milliamp/cm width. Spinco Model R paper electrophoresis apparatus was used.† The sample size was 0.006 ml of serum.

At the end of 16 hours, the paper strips were heated at 125°C for 30 minutes, fixed and stained in a 1% methanolic bromphenol blue solution(6). Prior to scanning in a Spinco Model RB Analytrol integrating densitometer, the dried strips were exposed to ammonium hydroxide vapor. A B-5 cam and 500 mμ interference filters with a 1.5 mm slit width were used. Total serum proteins were determined by the biuret method(7).

Results. Results of the electrophoretic de-

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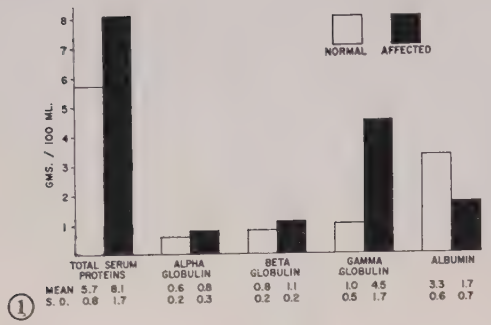

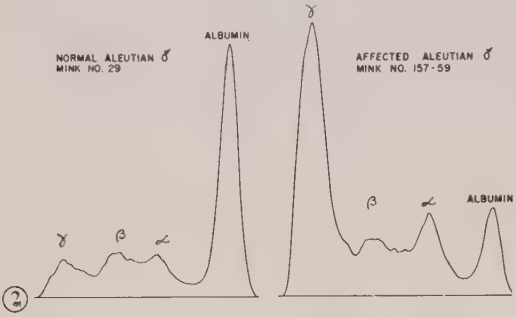

FIG. 1. The mean serum protein values of normal and Aleutian disease affected mink.

FIG. 2. Zone electrophoresis or normal and affected Aleutian mink serum.

terminations are summarized in Fig. 1. Total serum proteins and gamma globulin showed an absolute increase and the albumin showed an absolute decrease in the affected group. The differences in these fractions were highly significant using the "t" test. Examples of individual normal and abnormal patterns are shown in Fig. 2. Mink which appeared clinically healthy but later proved to have lesions consistent with this disease also showed abnormalities in the serum proteins. No animals which were normal at necropsy exhibited hypergammaglobulinemia. Normal mink observed in this experiment, regardless of genotype, had relatively the same serum protein levels.

Discussion. There were distinct differences in electrophoretic patterns and quantitative values of the serum proteins, especially gamma globulins, of mink affected by Aleutian disease when compared with those of healthy animals.

In preliminary trials to reproduce the disease experimentally, the authors have injected mink which were homozygous for the Aleutian gene as well as mink which were not homozygous for the gene with a formalinized tissue suspension. The source of tissue was a homozygous Aleutian mink which had lesions of the disease. Hypergammaglobulinemia as well as lesions consistent with Aleutian disease were produced in those animals which were homozygous for the Aleutian gene.

Mink with the naturally occurring disease have proteinurea and protein casts in the renal tubules. The result would be the loss of serum protein. Even though there is such a loss, total serum protein values in the diseased mink are raised. Therefore, there must be sufficiently increased serum protein production to compensate for the loss and yet raise the total amount of protein in the serum.

The finding of hypergammaglobulinemia in mink with plasmacytosis and vasculitis stimulates speculation that there may be similar pathogenetic factors in Aleutian disease of mink and such conditions as polyarteritis, lupus erythematosis, and other connective tissue diseases of man.

Summary. The electrophoretic patterns of normal and Aleutian disease affected mink have been determined. The serum from diseased animals had increased total serum proteins, increased gamma globulin, and decreased albumin.

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Relation of Mammotropes to Mammary Tumors. V. Role of Mammotropes in Radiation Carcinogenesis.* (26796)

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It has been well established that estrogenic hormones and the pituitary gland play a major role in development of mammary gland, in initiation and maintenance of growth of mammary tumors, and that in adults these actions of estrogens upon the mammary gland are mediated by a pituitary cell called mammotrope (Mt), which secretes mammary gland stimulating and growth promoting hormones(1). Enhancement of induction of mammary tumors (MT) by these hormones (MtH) in rats x-rayed with 200r or 400r over entire body, from 9% to 76%, within 7 months has been reported(2). It seemed desirable to confirm and extend this work using smaller doses of x-rays which alone will produce no MT within 7 months.

Materials and methods. The strain of rats (W/Fu) used and general procedures have been described(2,3). The rats were 2 to 3 months old when irradiated. The factors of x-irradiation were: 250 Kvp; 30 ma; filtration 1/4 Cu. 1.0 Al; distance 50 cm; air dose 150r/min. The rats were irradiated under light Nembutal anesthesia. As a source of steady supply of a natural MtH, transplantable mammotropic pituitary tumors (MtT) (Strains W-5 and W-6) were used. Strain W-5 was induced by radiation(2). Strain W-6 occurred in an old W/Fu female. MtT was grafted subcutaneously on back; when it reached about 3 cm in greatest diameter, most of it was resected to prevent death from a large tumor. The rats were sacrificed when a MT was palpable, and the MT and several mammary glands were examined microscopically. The ovaries of a small number of rats were removed 80 days after irradiation when the grafted MtT was not yet palpable. The experiments were terminated 7 months after irradiation when the rats were about 9 to 10 months old.

* Supported by Atomic Energy Commission and Nat. Cancer Inst.

Results. No MT occurred in 20 normal controls, in 23 rats treated with MtH alone and in 17 rats given 50r alone. Only 1 of 16 rats given 150r alone developed a MT (adenocarcinoma) at 202 days after irradiation. In contrast, 22 of 32 rats receiving both MtH and x-rays developed MT: 8 (53%) received 50r and 14 (82%) received 150r (Table I).

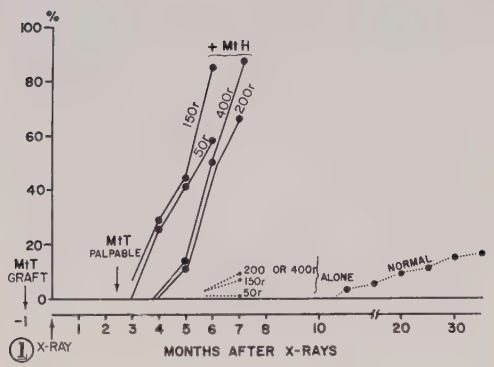
Fig. 1 shows the cumulative incidence of MT, including previous findings with 200r and 400r. The figure shows that onset of MT was slightly delayed when larger radiation doses were given. This apparent paradox can be readily explained. As the figure shows, the grafted MtT was also irradiated and it suffered from the large doses, its latency was prolonged and, consequently, these rats received less hormone than those given smaller doses of radiations. In general, MT became palpable earlier in those rats in which the MtT, that supplied the hormone, appeared sooner. Fig. 1 also shows that the spontaneous incidence of MT in females of this strain was 16% in 196 females observed up to 32 months of age and that all spontaneous tumors were fibroadenomas(3).

The types of MT and their latency are shown in Table II. Most MT induced by radiation alone were fibroadenomas; most induced by MtH plus radiation were adenocarcinomas. In general, induced carcinomas appeared earlier than fibroadenomas, as was already noted by Bond *et al.*(4).

Transplantation assay for autonomy. Most MT induced with 3-methylcholanthrene were

TABLE I. Mammary Tumor Induction by X-rays and MtH in Female W/Fu Rats.

Treatment	No. in group	MT	
		No.	%
None	20	0	
MtH	23	0	
50r	17	0	
150r	16	1	6.2
50r + MtH	15	8	53
150r + MtH	17	14	82



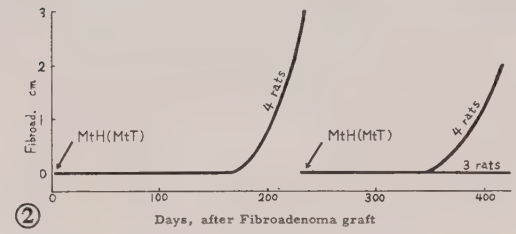


FIG. 1. Cumulative incidence of MT induced by X-rays and MtH in female W/Fu rats.

FIG. 2. Dependence of growth of grafted fibroadenoma on MtH.

autonomous but responsive to hormones(5). Tumors induced with large doses of this carcinogen, transplanted in successive passages, soon gave rise to fully autonomous variants; whereas, those induced with a single small dose of the carcinogen plus MtH remained fully MtH dependent(6). In light of this information, 3 radiation- plus MtH-induced tumors were tested for hormone responsiveness. All 3, a fibroadenoma and 2 adenocarcinomas, were found to be highly hormone responsive (Table III). They grew in all MtH-treated female rats, in few normal females, and in no normal males. The latent periods of grafted fibroadenomas were about 6 months and of the adenocarcinomas 2 months.

TABLE III. Transplantation of MT Induced by X-rays and MtH in W/Fu Rats.*

Tumor grafted	Recipient ♂	♀	♀ + MtH
Fibroadenoma W-1	0/4	1/8	4/4
Carcinoma W-19	0/5	1/8	4/4
" W-20	0/5	2/11	6/6

* Grafts from original tumors; sub-passages are in progress.

The dependence of growth of the fibroadenoma on MtH is shown in Fig. 2. This tumor was grafted in 11 female rats, 4 of which received MtH (by MtT graft) immediately after the graft, and tumors became palpable in the latter after about 180 days. About 230 days after the fibroadenoma graft, MtH was administered to 4 of the 7 remaining rats; all of these developed palpable tumors 140 days later. The 3 rats not receiving MtH were sacrificed 405 days after graft and carefully autopsied. No tumors were found. This finding is similar to that with 3-methylcholanthrene-induced hormone-dependent carcinomas which could be fully controlled by ovariectomy or hypophysectomy and resumed vigorous growth after administration of MtH(5). The present work indicates that MT induced by a combination of MtH and radiation can also be highly hormone (MtH) responsive.

TABLE II. Type and Latency of MT Induced by X-rays and MtH in Female W/Fu Rats.

Treatment	No. with MT/No. in group	Type of tumor Carcinoma	Fibroad.	MT latency after x-ray (month) 3	4	5	6	7
150r	1/16	1						1
200r*	1/11		1					(1)‡
400r*	1/11		1					(1)
MtH + 50r	8/15	6	2		3	2 (1)	1 (1)	
MtH + 50r + Ovex§	1/4	1				1		
MtH + 150r	14/17	11	3	2	3	1 (2)	5 (1)	
MtH + 150r + Ovex§	2/4	1	1					1 (1)
MtH + 200r*	6/9	4	2			(1)	3	1 (1)
MtH + 400r*	7/8†	2	6			1 (1)	(3)	1 (2)

* Reported earlier(2) without specification as to latency and type.

† One of these rats had 2 tumors, a fibroadenoma and an adenocarcinoma, listed separately.

‡ Figures in parentheses are fibroadenomas.

§ Ovex = bilateral ovariectomy, performed 80 days after x-irradiation.

TABLE IV. Effect of Ovariectomy on MT Induction by X-rays and MtH.

Treatment	No. of rats	No. with MT	Tumor latency (days)
X-ray*	6	0	
X-ray + MtH	6	3	90, 125, 145 (120)‡
X-ray + Ovex†	8	0	
X-ray + MtH + Ovex	8	3	145, 210, 210 (188)

* 50r or 150r, over entire body.
† Bilateral ovariectomy, performed 80 days after x-irradiation.
‡ Figures in parentheses indicate mean latency.

In a small experiment, 8 irradiated plus MtT grafted rats were ovariectomized 80 days after irradiation when MtT was not yet palpable. Ovariectomy seems to slow down MT development and may also prevent MT formation in some rats (Table IV).

Discussion. Two strong factors have long been recognized in mammary tumorigenesis: estrogenic hormones which exert their influence by way of the pituitary Mt(1) and carcinogens which cause a permanent modification of the mammary epithelium. Earlier studies have shown that ionizing radiations stimulate Mt(2), the supplier of MtH that promotes MT development. Thus, radiations have a double action: they "cancerize" mammary epithelium and stimulate its promoter, Mt.

The relative role of hormones and carcinogens in induction of mammary tumors has been fully discussed elsewhere(7). The observations here presented suggest that small doses of radiations can create latent neoplastic cells and that these cells form progressively growing tumors only when the hormonal stimulus (MtH level) is elevated. Thus, it seems that the dependent tumorous mammary epithelium has a greater sensitivity to MtH than the normal epithelium. A similar heightened sensitivity to estrogens of dependent tumorous pituitary Mt, in comparison to normal Mt, is indicated by studies of Clifton(8) by means of grafting these cells beneath the kidney capsule. Formation of such dependent neoplasms is a unique type of carcinogenesis in which tumor formation is brought about by a mild permanent modification of the cell in presence of elevated levels of its stimulant.

Experiments on the degree of mammary gland stimulation by MtH, as indicated by incorporation of tritiated thymidine, are in progress in our laboratory. B. H. Sells and B. Messier found that under stimulation of MtH (by a small graft of MtT), the mammary tissue incorporates twice as much tritiated thymidine per gram as unstimulated mammary tissue and total uptake of tritiated thymidine in stimulated glands is nearly 10-fold normal (unpublished data).

These and other studies(7) led us to the generalizations that physiologic doses of hormones are not carcinogens and that small, so-called subcarcinogenic doses of x-rays as well as chemical carcinogens and virus can produce many latent neoplastic cells and that the latter can be activated by proliferative stimuli of which hormones are outstanding.

How long latent tumor cells can survive and what the relation is between their numbers and the dose of radiation remains to be studied. Conceivably, there is no threshold for the "carcinogenic" alteration which may be "linear" but it is unlikely that tumor formation follows the same "linearity." The work discussed indicates that to bring about a tumor formation, promoting factors, such as MtH, are needed. The availability of the latter and the magnitude of the radiation damage determine the formation of tumors by altered cells.

Summary. 1. X-rays (50r) and mammotropic hormones (administered in form of isologous functional mammotropic pituitary tumor grafts) alone produced no mammary tumors in female W/Fu rats within 7 months of treatment. In contrast, 53% rats treated with both, developed mammray tumors. As with chemical carcinogen-induced tumors, administration of mammotropic hormones restored inhibition of mammary tumor development by ovariectomy. 2. Most mammary tumors induced by radiation plus mammotropic hormones were adenocarcinomas; a few were fibroadenomas. 3. Three induced tumors tested were found to be highly mammotropic hormone responsive: they did not grow in males and grew in females uniformly

only when stimulated with mammotropic hormones. 4. Grafted fibroadenoma cells remained latent in many rats until administered mammotropic hormones brought about their rapid growth. Administration of this hormone could be delayed as long as 7½ months. 5. It is concluded that radiations bring about an irreversible modification of some cells, depending on severity of the dose; mammotropic hormones are not carcinogens but, as promoters of mammary epithelium, promote carcinogenesis and growth of some tumors.

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Erythrocyte Survival Studies in Experimental Molybdenosis of Sheep.* (26797)

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The toxicity of a high molybdenum intake in the diet has been extensively studied. In cattle, the effects of toxicity include severe diarrhea, emaciation, loss of coat color and anemia. In sheep on similar intakes of molybdenum, the clinical picture appears to be principally associated with signs of a copper deficiency. The relationship between molybdenum and copper metabolism together with the influence of inorganic sulfate has been recently reported by Dick(1). It was shown that the symptoms of copper deficiency in sheep develop only in presence of increased molybdenum and sulfate in the diet. Similar molybdenum sulfate-copper relationships in cattle have been reported(2). Experimental molybdenosis has not been shown to influence hematology in sheep(3) or cattle(2) even though an anemia is associated with the natural condition in cattle.

This report presents data concerning the life span of erythrocytes in experimental molybdenosis of sheep using the direct glycine-2-C^{14} method. Erythrocyte life span for normal sheep using this method has been previously reported(4).

Materials and methods. Two female lambs (6161, 6170), one year of age and weighing 28 and 32 kg, respectively, were used. Each lamb was initially placed on a daily oral intake by capsule of 45.4 mg of molybdenum as sodium molybdate for 54 days. Since no clinical symptoms of toxicity appeared, daily intake was doubled for the next 35 days, during which time severe diarrhea developed. Daily intake was then returned to the original amount for 10 days. At this time, 100 μc and 150 μc of glycine-2-C^{14} were injected intravenously into lambs 6161 and 6170, respectively. Molybdenum administration was continued throughout the trial.

The procedures employed for determination of median survival time for erythrocytes in sheep have been reported(4,5). Median survival times ($t_{1/2}$), of the erythrocytes of the sheep in this study were estimated from their survival probability function, $p(t)$, the probability that an erythrocyte will have a survival time greater than t.

Plasma and liver copper levels were determined in the sheep at termination of the trial

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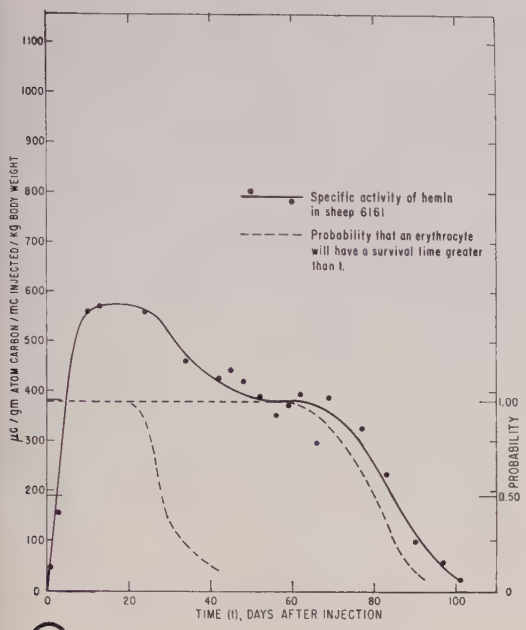

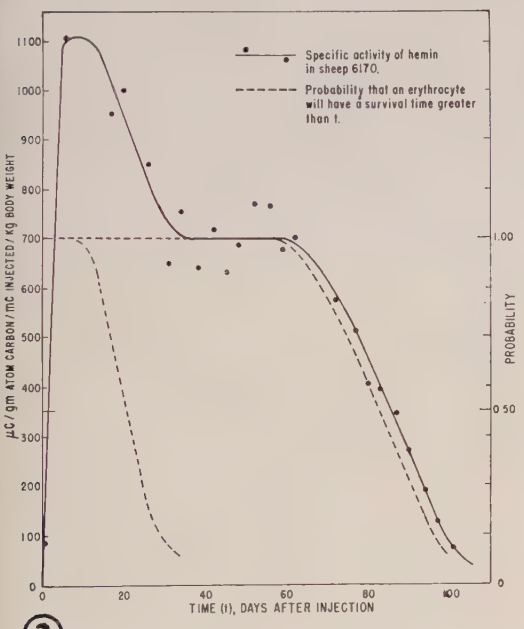

FIG. 1. Specific activity of hemin and survival probability function in a sheep with molybdenosis.

FIG. 2. Specific activity of hemin and survival probability function in a sheep with molybdenosis.

by methods previously reported(6). Hematological determinations were performed by standard methods.

Results and discussion. The specific activities of hemin and their respective survival probability functions are presented in Fig. 1 and 2. The 2 distinct plateaus of the specific activity curves are not in accord with the single plateaus observed in the curves of normal sheep(4). A similar finding of 2 plateaus in the life span curve has been reported for the normal aoudad sheep(5). This was interpreted as the existence of 2 separate populations of erythrocytes with different survival times. Similar calculations for the curves obtained in the present study resulted in median erythrocyte survival times of 28 and 80 days for sheep 6161, and 20 and 84 days for sheep 6170. The second population of red cells with longer survival times in each sheep was comparable to that found in normal sheep(4).

We attribute the first decrease in specific activity of hemin to death of one population of erythrocytes and the second decrease to death of a second population. It can be estimated(5) that in these sheep, 30-40% of the total populations exhibited the shorter survival times. In rabbits recovering from experimentally produced anemia, it has been reported(7) that some of the cells had a shortened life span. This finding was also observed in the rat(8) under similar conditions. It was suggested that new red cells produced rapidly in response to the anemia were functionally inadequate and thus were short lived. This would imply that the daily turnover of erythrocytes in these sheep should be greater than normal. However, erythrocyte and leukocyte counts performed on the sheep were within normal limits during the entire course of the experimental trials. Some polychromasia was observed in the peripheral blood smears but myeloid-erythroid ratios of bone marrow samples were also within normal limits.

The levels of plasma copper obtained in these sheep were 22 and 43 μg % for 6161 and 6170, respectively. These values are below normal values of 58-160 μg % reported for sheep(6). Liver copper levels were 4.3 and 5.7 mg/100 g wet weight for sheep 6161 and 6170, respectively, and are near the lower limit of the 4-25 mg/100 g wet weight reported for normal sheep(6). Dick(1) has

postulated that plasma copper levels in sheep fall due to depletion of copper stores as a result of prolonged molybdenum and sulfate administration. Sulfate intake was not measured in the trials reported here. It would appear, however, that sulfate intake in these sheep was sufficient, together with the added molybdenum to lead to a copper depletion. The requirement for copper in synthesis of heme is known and Anderson and Tove(9) have recently demonstrated a direct role for copper in this synthesis. Copper deficiency in sheep characteristically exhibits a demyelinating lesion in the central nervous system of lambs while only under extreme conditions will anemia occur in the ewe. Anemia is also a characteristic of molybdenosis in non-ruminant animals and has been attributed to inability of these animals to utilize stored copper(10). Bush, *et al.*(11), found a shortened red cell survival using Fe^{59} in copper deficiency of swine and attributed this to subnormal levels of copper within the red cell. If the phenomenon were similar in these sheep, one might expect to find a shortening of survival time of the entire red cell population rather than 30-40% as found in the present study, unless only a marginal deficiency had occurred.

Summary. Median survival times of the erythrocytes of sheep in experimental molybdenosis were determined using glycine-2-C^{14}. The existence of 2 distinct populations of erythrocytes was observed in each sheep. Median survival times for the first erythrocyte populations in 2 sheep were 20 and 28 days, with 80 and 85 days for the second populations.

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Quantitative and Temporal Studies on Effect of Dexamethasone on Corticosterone Secretion in the Rat.* (26798)

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Depression of plasma or adrenal corticosterone concentration by exogenous steroid administration is well known. Pfeiffer and co-workers suggested the substitution of dexamethasone-blocked animals for hypophysectomized animals in assays of ACTH using peripheral plasma corticosterone concentration as the index of adrenocortical response (1). Peron and Dorfman found that measurement of adrenal corticosterone content provided a useful method of assaying the ACTH-suppressing potency of certain corticoids(2). This report describes the suppressive influence of dexamethasone on adrenal corticosterone secretion rate in the rat.

Methods. Adult male Sprague-Dawley rats weighing 200-250 g were used following a 4 to 10 day acclimatization period in the laboratory. Dexamethasone-21-phosphate†

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(stock concentration 4 mg/ml) was diluted daily with physiological saline to bring final injection volumes to 0.5 ml. Control animals received 0.5 ml of physiological saline. All injections were made subcutaneously in the unanesthetized animal. At selected intervals after injection the adrenal venous effluent was sampled. The animals were anesthetized with ether, heparin (100 units in 0.1 ml) was administered *via* the femoral vein, and laparotomy was quickly performed. A #27 gauge needle, fixed on a tuberculin syringe, was inserted through the left renal vein wall opposite the confluence of the left adrenal vein and threaded into the orifice of the adrenal vein to the point of confluence of small diaphragmatic venules. The adrenal effluent was collected for exactly 3 minutes. To avoid backflow through conjoining veins, steady, gentle withdrawal of the syringe plunger was continued through the collection time. The volume of blood obtained usually ranged from 0.6 to 0.9 ml. To minimize errors due to occasional faulty collection, samples not falling within this volume range were discarded. The entire procedure required 6 to 7 minutes per animal. After stoppering the syringe barrel with a small rubber cap it was used as a centrifuge tube, and the plasma was separated and frozen. Plasma corticosterone was determined by a modification of the fluorometric method of Silber and co-workers(3). Silber's correction for "blank" fluorescence of rat plasma was used in calculation of steroid determinations. In preliminary experiments to determine the fluorescence of dexamethasone-21-phosphate, plasma from hypophysectomized rats obtained one day following hypophysectomy was used.

TABLE I. Fluorescence Readings of Steroids in Hypophysectomized Rat Plasma.

Steroid	μg added/ml	Relative fluorescence
Control	—	6 11
Corticosterone	.2	29
	.4	62
	.8	128
	1.6	225
Dexamethasone	.2	7
	.4	7
	.8	11
	1.6	7
	4000.0	11

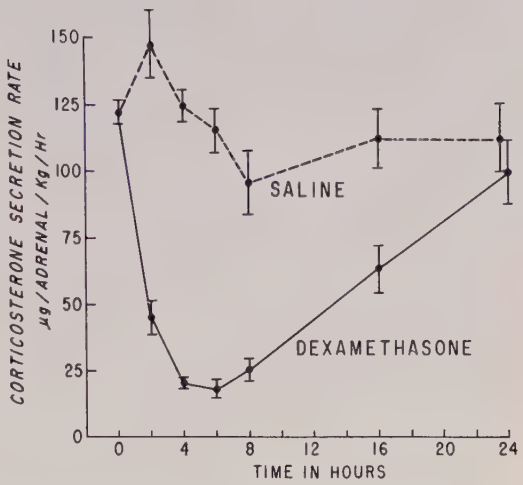

FIG. 1. Comparative effects of a single inj. of dexamethasone-21-phosphate, 100 μg/kg body wt, and saline on the rat corticosterone secretion rate. Each point represents mean secretion rate of 6 or more animals. Stand. errors indicated by vertical bars.

Results. Dexamethasone-21-phosphate itself did not fluoresce under the conditions of the experiment. Table I summarizes fluorescence data obtained after adding dexamethasone-21-phosphate to hypophysectomized rat plasma. The steroid did not alter fluorescence in concentrations up to 4 mg/ml. The steroid was also given intravenously to rats immediately before sampling the adrenal effluent. Corticosterone secretion rate of the dexamethasone-treated animals (120 ± 9.4‡ μg/kg/hr; n = 5) similar to that of untreated controls (123 ± 5.3 μg/kg/hr; n = 17).

Fig. 1 depicts corticosterone secretion rate determinations obtained in dexamethasone-treated (100 μg/kg body weight) and control animals at various intervals following a single subcutaneous injection. Maximal suppression of corticosterone secretion rate occurred 4 to 8 hours following administration of the steroid, and recovery to pretreatment values was nearly complete by 24 hours. Corticosterone secretion rate of saline-injected controls remained at approximately the same values throughout the experiment, suggesting

‡ Values expressed as mean corticosterone secretion rate/kg/adrenal/hr ± standard error.

TABLE II. Dose-Response Effect of Dexamethasone on Rat Corticosterone Secretion Rate 4 Hours after Injection.

Dose, μg/kg body wt	No. of rats	Corticosterone secretion rate*
0	17	123 ± 5.3
10	10	101 ±12.2
40	10	49 ± 6.7
100	10	21 ± 2.6
400	10	23 ± 3.4

* Values expressed as mean μg/kg/adrenal/hr ± stand. error.

that the potential stimulating effect of the injections themselves did not influence secretion rates.

Table II summarizes dose-response data obtained 4 hours after administration of a single dose of dexamethasone-21-phosphate. Maximal suppression of corticosterone secretion rate was produced by 100 μg/kg body weight.

Repeated determinations of corticosterone secretion rate were made in a few animals. Immediately following the initial sampling, 10 mg of hexadimethrine bromide§ was given intravenously to counteract the anticoagulant effect of heparin, and the abdominal incision closed. One week later the adrenal venous effluent was resampled. Minor adhesions were usually encountered at the second operation, but did not prevent the sampling procedure in any animal. Corticosterone secretion rate at the second sampling (131 ± 12 μg/kg/hr; n = 6) was similar to that of untreated controls.

Discussion. Reported values of corticosterone secretion rate from one adrenal gland in the ether-anesthetized rat have been in fairly good agreement despite the variety of methods employed. Collections times have ranged from 15 minutes(4,5) to 1.5 hours(6), but secretion rates have ranged from approximately 100 μg/kg/hr(4) to 150 μg/kg/hr (6). This may reflect near maximal corticotropin release due to ether and/or the stimulus of surgery inasmuch as exogenous corticotropin results in only slight augmentation of the "resting" secretion rate(4).

The degree and duration of suppression of the corticosterone secretion rate following a single dose of dexamethasone agrees closely with the suppression of peripheral plasma corticosterone concentration found in response to exogenous steroids(1). Rate of fall of the secretion rate and onset of maximum suppression also occur at approximately the same time that adrenal steroid secretion rate reaches a minimum in hypophysectomized dogs(7).

The relatively large concentration of corticosterone present in the adrenal effluent of untreated control animals (about 3 μg/ml) compared with peripheral plasma concentration (about 0.2 μg/ml) provides greater amounts of steroid for assay. This technic may be adaptable either to corticotropin assays or to assays of corticotropin-suppressing potency of other non-fluorescing corticosteroids.

Summary. The rat corticosterone secretion rate determined fluorimetrically from 3-minute samples of adrenal effluent (123 ± 5.3 μg/kg/adrenal/hr) agrees well with values reported for more extended collection periods and analyzed by other methods. Dexamethasone-21-phosphate, a non-fluorescing, corticotropin-suppressing, synthetic steroid was found to produce maximal suppression of the corticosterone secretion rate 4 to 8 hours following a single dose. The technic described may be applied to assays of corticotropin itself or assays of corticotropin suppressing activity of other non-fluorescing agents.

§ Polybrene, Abbott.

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Effect of L-Histidine on Creatine, Histidine, and 1-Methylhistidine Excretion of Normal and Vit. E-Deficient Rabbits.* (26799)

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It has been shown(1,2) that Vit. E-deficient diet caused rabbits to excrete 1-methylhistidine in large amounts. Appearance of this amino acid in urine generally precedes that of creatine making it one of the earliest detectable symptoms of nutritional muscular dystrophy. McManus(1) has shown a diminished concentration of muscle carnosine and anserine suggesting an abnormality in the metabolism of these dipeptides. She believes that Vit. E-deficiency results in a hydrolysis of muscle anserine to beta-alanine and 1-methylhistidine as well as a decreased synthesis of anserine.

Regardless of the causative mechanism, the loss of large amounts of 1-methylhistidine might itself produce secondary effects such as a significant drain on the body's reserve of the essential amino acid histidine. Our studies describe the effect of dietary L-histidine on excretion of creatine, histidine, and 1-methylhistidine of normal and Vit. E-deficient rabbits.

Methods. Twelve male rabbits, divided into 4 equal groups, were fed the following diets *ad lib.*: A. Dystrophy-producing diet of Young and Dinning(3) plus 8 mg alpha-tocopherol acetate thrice weekly: B. Dystrophy-producing diet containing 1% L-histidine plus 8 mg oral alpha-tocopherol acetate thrice weekly: C. Dystrophy-producing diet: D. Dystrophy-producing diet containing 1% L-histidine. When histidine was incorporated into the diet, an equivalent weight of sucrose was deducted.

Twenty-four hour urine samples were collected occasionally in a rabbit metabolism cage. Creatine and creatinine were determined by the method of Folin(4) while quantitative determinations of histidine and 1-methylhistidine were made by Dowex-50 chromatography using the method of Tallan *et al.*(5).

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Results. Urinary histidine and 1-methylhistidine values and creatine/creatinine ratios are shown in Table I. Values are expressed as μM/24 hr for the 2 amino acids. Rabbits of Group A excreted less histidine than those of Group B although the difference was not significant. No animals of Group A excreted any detectable amounts of 1-methylhistidine. Rabbit 6 of Group B did excrete this amino acid after 31 days on the diet. This same animal exhibited a creatine/creatinine ratio of 0.83 after 41 days on the diet while the remaining rabbits of both groups had insignificant ratios throughout the experiment.

Animals of Group C excreted significantly smaller amounts ($p<0.01$) of histidine than those animals on Diet A. One rabbit of Group C excreted none while the other 2 excreted less than half that of animals on Diet A. In contrast, those on Diet D excreted similar amounts to those on Diet B until about 3 weeks when histidine was no longer detectable. Animals on Diet C excreted large amounts of 1-methylhistidine within 2 weeks after being placed on the deficient diet. The amounts generally increased until death occurred. Rabbits fed Diet D excreted smaller amounts ($p>0.01<0.05$) of 1-methylhistidine than those on Diet C, *viz.*, after 3 weeks, an average excretion of 59 μM *vs* 165 μM. Animals on Diet C also excreted less histidine ($p>0.01<0.05$) than those on Diet D as well as exhibiting creatinuria within 2 weeks after being placed on the deficient diet, in contrast to Group D. Just before death, the creatine/creatinine ratios of those on Diet C rose to 4.7, 6.4, and 2.2 respectively, while animals on Diet D, at the same dates, showed ratios of 1.1, 0.2, and 1.5.

Discussion. Perhaps the most important point to be made from these experiments is that animals receiving an E-deficient diet containing 1% histidine excreted less urinary 1-methylhistidine than those not receiv-

TABLE I. Effect of Dietary L-histidine on Urinary Histidine and 1-methylhistidine, and Creatine/Creatinine Ratios of Normal and Vit. E-Deficient Rabbits. Values expressed as μM/24 hr.

Diet		Rabbit No.	Days on diet	Histidine	1-methyl-histidine	Creatine / Creatinine
A	Vit. E-deficient + vit. E	1	17	3.9	.0	.10
		1	35	7.5	.0	—
		2	15	6.6	.0	.04
		2	32	17.6	.0	.03
		3	46	2.9	.0	—
B	*Idem* + 1% L-histidine	4	12	14.0	.0	—
		4	32	13.4	.0	.36
		5	10	8.0	.0	.08
		5	13	19.2	.0	.10
		6	31	10.0	3.3	.35
		6	41	10.7	24.2	.83
C	Vit. E-deficient	7	16	.0	64.2	1.44
		7	19	2.6	73.5	4.71
		8	12	.0	176.8	.37
		8	22	.0	292.7	1.45
		8	24	.0	406.6	6.42
		9	13	2.0	222.6	.98
		9	22	1.9	128.0	2.19
D	*Idem* + 1% L-histidine	10	12	16.6	18.0	—
		10	20	.0	38.7	.28
		10	25	.0	120.9	1.14
		10	31	.0	231.6	.80
		11	9	8.4	.0	—
		11	24	.0	57.4	.18
		11	26	13.3	118.7	.22
		11	29	45.4	75.0	1.54
		12	9	9.0	.0	—
		12	16	31.4	35.3	.13
		12	23	.0	79.7	1.53

ing the supplement. Furthermore, the additive markedly reduced creatinuria and prolonged longevity an average of 16 days, or about 75% as compared to those on the deficient diet alone.

Also, animals fed normal diet did not excrete methylhistidine. This would agree with some findings(2,6); however not with other workers(1,5,7) who found a low content of 1-methylhistidine in normal rabbit urine. It should be stressed that rabbit 6 did excrete this derivative while receiving alpha-tocopherol, but also had some creatinuria. This would infer a mild dystrophy, although at no time were physical symptoms obvious.

It might be expected that rabbits on Diet B would excrete more histidine than those not receiving the supplement on Diet A since both sets consumed similar amounts of diet with the former thus receiving about 0.4 g per day more histidine than did the latter group. Results further indicate that animals on an E-deficient diet excreted little histidine in the urine but rather massive amounts of 1-methylhistidine. The latter finding would agree quantitatively with those of Fink *et al.* (2) that dystrophied rabbits excreted as much as 400 μM/24 hr/kg body weight of the methyl derivative. Their results were about 5-fold those reported elsewhere(1).

McManus(1) has shown that normal and dystrophied muscle are devoid of carnosinase activity, and suggested that anserine could be hydrolyzed elsewhere to cause methylhistidinuria in E-deficient animals. This would deplete histidine from the various metabolic pools and could explain Tallan's(8) findings of a decrease in free histidine of muscle of severely E-deficient rabbits. The possibility also exists that there is an accelerated rate of protein breakdown to furnish needed histidine. This would account for the significant rise in free amino acids of various tissues as shown by Smith and Nelson(9).

It is conceivable that supplementation of the deficient diet with histidine would retard the dystrophic process by maintaining a high level of histidine in the metabolic pool. This would result in a diminished creatinuria and methylhistidinuria.

Summary. 1. Effects of L-histidine on excretion of 1-methylhistidine, histidine, and creatine of normal and Vit. E-deficient rabbits have been studied. 2. L-histidine caused an increased excretion of histidine from animals on a normal and Vit. E-deficient diet. 3. Three rabbits on a normal diet and 2 of 3 on a normal diet + L-histidine failed to excrete 1-methylhistidine in the urine. 4. Vit. E-deficient animals excreted small amounts of histidine, but large quantities of 1-methylhistidine and creatine. 5. Vit. E-deficient rabbits fed 1% L-histidine excreted less 1-methylhistidine and creatine, but increased amounts of histidine. 6. 1% L-histidine was shown to prolong the lives of Vit. E-deficient rabbits.

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Studies on Platelets XXIV. Analysis of Some Platelet Antigens by Complement Fixation Test.* (26800)

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Platelets exhibit species specificity(1) and very likely cell(2) and blood group(3,4) specificity. Specific antigens proper to platelets only may also be found(5,6). Evidence for organ specificity of platelets has also been presented. Thus, injection of serum from patients with chronic idiopathic thrombocytopenic purpura may induce thrombocytopenia in dogs(7) and rabbits(8), an effect which is abolished if the serum is previously absorbed with human platelets. Also, the platelet agglutinins found in human idiopathic thrombocytopenic purpura are absorbed by monkey platelets(9), while antiguinea pig platelet rabbit serum agglutinates human platelets as well(10). Finally, there is a common antigenic structure of platelets obtained from man, goat, sheep, horse and pig(11). Most of the previous work *in vitro,* however, has been carried out using agglutination procedures. This report presents an analysis of the organ specificity of platelets using a more reliable technic, namely complement fixation.

Materials and methods. (a) *Preparation of platelets.* Blood was collected from the antecubital vein (man) or jugular vein (horse, cow and dog) into plastic bags with ACD solution or into silicone-coated bottles containing 0.1 volume of 1% Sequestrene-Na_2 solution. Platelets were separated and washed 5× with isotonic saline by standard technic(6). Large amounts were frozen at −4°C until used for immunization purposes.

(b) *Preparation of platelet antigen for complement fixation test.* A platelet button

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grossly free of red cells was resuspended in isotonic saline to contain from 350,000 to 500,000 platelets/ml and frozen at –2°C for 1 hour. After thawing, the suspension was ground in a glass mortar, shaken for 10 minutes and centrifuged at low speed for a few minutes to remove the larger particles. The concentration which gave complete complement fixation with standard homologous antiserum without showing anticomplementary activity corresponded to a density of 0.3 to 0.4 O.D. at 400 mμ by spectrophotometric reading. A 1/10 dilution of the antigen in isotonic saline solution was used in the absorption technics (see below).

(c) *Preparation of platelet antigen for injection into rabbits.* An equal volume of alum precipitate was added to the ground platelet suspension prepared as in (b) and the mixture shaken gently before use. Each rabbit received 5 ml of mixture into the gluteal muscle 3 times a week until a good titre was obtained (in about 4 weeks).

(d) *Human serum* was obtained from healthy individuals and inactivated at 56°C for 30 min. A 1/1000 dilution of serum in isotonic saline solution was optimal for performance of the complement fixation test.

(e) *Complement.* Commercially available lyophilized guinea pig serum was used as source of complement throughout. The serum was reconstituted and stored in 1 ml aliquots at –20°C until used.

(f) *Complement fixation test* was carried out by the method of antibody dilution. Inactivated antiserum was diluted serially with 0.9% NaCl solution. Aliquots of 0.2 ml of each dilution were added to equal volumes of antigen. Two units of guinea pig complement were added. The mixtures were incubated at 37°C for one hour. A hemolytic system (5 units of sensitized 2% sheep cells) was then added (0.2 ml volume). Results were read after one hour incubation at 37°C, with the confirmation of complete hemolysis in the necessary control tubes.

(g) *Absorption technics.* The ratio of absorbing antigen to antiserum depended on the particular agent used for absorption. When serum was used as the absorbing antigen, one volume of 1/50 diluted serum was mixed with one volume of 1/5 diluted antiserum. When platelets were used as the absorbing antigen, one volume of 1/10 diluted antigen was mixed with one volume of 1/5 diluted antiserum. In each case, the mixture was allowed to stand at room temperature for 2 hours and overnight at 4°C, then centrifuged at high speed for 30 min. The supernatant was carefully drawn off and considered to be a 1/10 dilution of the absorbed antiserum.

TABLE I. Complement Fixation Reaction of Antihuman Platelet Immune Rabbit Serum.*

Antigen	Antiserum (dilution) 20	40	80	160	320	640
Human platelet	0	0	0	0	0	1+
Horse "	0	0	1+	2+	3+	3+
Bovine "	0	0	0	1+	2+	3+
Dog "	0	0	1+	2+	3+	3+
Human serum	0	0	2+	3+	3+	3+
	After absorption with human serum					
Human platelet	0	0	0	0	0	1+
Horse "	0	0	1+	2+	3+	3+
Bovine "	0	0	1+	2+	3+	3+
Dog "	0	0	1+	2+	3+	3+
Human serum	3+	3+	3+	3+	3+	3+

* 0 = no hemolysis; 1+ = weak hemolysis; 2+ = strong hemolysis; 3+ = complete hemolysis.

Results. Table I indicates that antihuman platelet rabbit serum reacted with horse, bovine and dog platelets, although at a lower titer than with the main antigen (human platelets). Silber *et al.*(12) have recently stated that, using a fluorescent antibody method, dog and pinea pig platelets also react with fluorescent rabbit antihuman platelet serum, although with less intensity. In our experiments, the antihuman platelet rabbit serum also reacted with human serum. To eliminate the possibility that the reaction might have been due to presence of serum proteins in the platelet antigen, the antihuman platelet immune rabbit serum was absorbed with human serum. It reacted with animal platelets with almost the same avidity before and after the absorption. Also, the absorbed immune serum reacted with human platelets in an undiminished titer, a finding of interest which suggests many speculations. Table II shows that absorption of antihuman platelet immune serum with heterologous platelets also reduced its activity against homologous platelets.

TABLE II. Absorption of Antihuman Platelet Immune Rabbit Serum with Heterologous Platelets.

	Before absorption with horse platelets					After absorption with horse platelets				
	Antiserum dilution					Antiserum dilution				
Antigen	20	40	80	160	320	20	40	80	160	320
Human platelet	0	0	0	0	1+	0	1+	2+	3+	3+
Horse "	0	0	1+	2+	3+	3+	3+	3+	3+	3+

Conclusion and summary. It is confirmed that platelets have organ specificity, possessing an antigen common to platelets of different species, as well as species specificity, since antiplatelet antibodies react more strongly with homologous than with heterologous platelet antigens.

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Antibody Production in Guinea Pigs Receiving 6-Mercaptopurine.* (26801)

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Widespread interest in the possibility of suppressing the immunological responses of animals by administration of chemicals has followed the reports of Schwartz *et al.*(1-3) that rabbits treated with 6-mercaptopurine (6-MP) during primary antigenic stimulation fail completely to produce humoral antibodies. Actually, several authors(4,5) have observed some antibody formation in 6-MP treated animals receiving antigen in Freund's adjuvant, but attribute the incomplete suppression to the intense stimulus afforded by this method of antigen administration. Evidence has now been obtained that, in the guinea pig, 6-MP does not even partly block the production of precipitating antibody to bovine gamma globulin (BGG) or development of delayed contact dermatitis to picryl chloride, though each material is administered without adjuvant.

Methods. To test its effect on production of circulating antibody, 6-MP (Purinethol, Burroughs Wellcome) was administered at 2 dose levels (9 mg/kg/day i.m. to 300-350 g guinea pigs; 40 mg/kg/day i.p. to 400-750 g animals) beginning 2 days before and continuing for 14 days after injection of antigen. Fresh solutions were prepared daily by dissolving the 6-MP in 1 N NaOH, adjusting at once to pH 9.0 with 0.1 M KH_2PO_4, and diluting to 10 mg/ml with distilled water.

Each test and control animal was bled from the heart prior to and 14 days following a single intraperitoneal injection of 5 mg of

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TABLE I. Precipitins to BGG Following Primary Antigenic Stimulation of 6-MP Treated Guinea Pigs.

Daily treatment	No. animals with 14-day anti-BGG titer* of: 0†	1/2	1/4	1/8	1/16	1/32	1/64	Total No. animals
9 mg/kg 6-MP, i.m.	3			2	2	1	3	11‡
None	2	1	1		2	2	2	10
40 mg/kg 6-MP, i.p.					2	2		4
pH 9.0 buffer, i.p.		1		1	1	1		4

* Dilution of serum before addition of antigen.
† 0 = no reaction in test with 1/2 dilution of serum.
‡ Four animals received 6-MP in 0.1 N NaOH instead of in pH 9.0 buffer.

BGG (Armour Fraction II) in 1 ml saline. Most of the animals were bled again 9 weeks later, immediately prior to and 5 days after a second 5 mg dose of BGG given subcutaneously. Serial dilutions of all sera were tested for antibody by mixing with an equal volume of 0.2 mg/ml BGG in 1% sodium acetate and reading for turbidity following incubation for 1 hour at 37°C and storage overnight at 4°C.

To study the effect of 6-MP on development of delayed contact hypersensitivity, 6 or 40 mg/kg/day was injected into 2 groups of albino guinea pigs beginning 2 days before and continuing for 9 days after intracutaneous inoculation of a total of 15 μg of picryl chloride (aqueous solution) into 6 nuchal sites. On the eighth day after antigenic stimulation, the 6-MP treated animals were compared, in respect to extent of contact hypersensitivity produced, with guinea pigs that had received only the picryl chloride inoculation. Reactions were evaluated 24 and 48 hours following dermal application of one drop of 1% picryl chloride in olive oil(6).

As a control on the effect of 6-MP upon production of circulating antibody by guinea pigs, the drug was injected (9 mg/kg/day i.m.) into each of 7 female rabbits, beginning 2 days before and continuing (in the case of 4 of the animals that survived the regimen) for 12 days following a single intraperitoneal injection of BGG (30 mg); 7 additional rabbits in the same weight range (2300-3800 g) received only the antigen. Animals were bled prior to administration of 6-MP as well as 12 days after injection of antigen, and serial dilutions of all sera tested for antibody content by the precipitin test and by agglutination of "tanned" BGG-coated erythrocytes according to the technic of Boyden.

Results. Table I shows that, following a single inoculation of BGG in saline, guinea pigs injected with either 9 or 40 mg/kg/day of 6-MP formed antibody as well as or perhaps better than animals that had not received the drug. Thus, precipitin titers of 1:8 or higher were found in 12 of 15 6-MP treated animals, as compared with 9 of 14 animals receiving no drug; precipitins were not detected in sera from 3 drug-treated animals or from 2 controls. Sera of all animals were negative prior to the primary antigenic stimulus.

Table II summarizes the secondary responses of a number of the above 6-MP treated and control guinea pigs to a further small dose of BGG given 9 weeks after primary stimulation. All but one of the animals receiving 6-MP prior to and concurrently with the second antigenic stimulus showed a rise in precipitin titer. There was no significant difference in extent of the anamnestic response of the 6-MP treated and control guinea pigs.

In separate experiments (Table III) 6-MP treated guinea pigs, inoculated with a minute amount of picryl chloride, responded with development of delayed dermal hypersensitivity to picryl chloride as pronounced as that seen in control animals.

In contrast to these observations in guinea pigs, and in keeping with the original data of Schwartz *et al.*(1-3), each of the 4 rabbits surviving the 6-MP treatment failed completely to produce antibody to BGG as judged by the negative results in precipitin tests with undiluted and diluted sera as well as in the more sensitive hemagglutination test employing BGG-coated erythrocytes (sera tested in dilutions as low as 1:10). Five of

TABLE II. Precipitins to BGG Following Secondary Antigenic Stimulation of 6-MP Treated Guinea Pigs.

Treatment	Bleeding, in relation to 2° BGG inj.	No. animals with anti-BGG titer* of: 0†	1/2	1/4	1/8	1/16	1/32	1/64	Total No. animals
6-MP‡	1 day before	3	5	1					9
	5 days after	1		1	2	4	1		9
None	1 day before	6	3						9
	5 days after			3	1	4		1	9

* Dilution of serum before addition of antigen.
† 0 = no reaction in test with 1/2 dilution of serum.
‡ Animals reinjected with 6-MP (9 mg/kg i.m. or 40 mg/kg i.p.) on 7 successive days, beginning 2 days before secondary antigenic stimulation.

the 7 control rabbits subjected to the same antigenic stimulus showed precipitins in dilutions of 1:4 to 1:16 and hemagglutinins to BGG-coated erythrocytes at titers of 1:1280 or greater; only one control animal failed to produce detectable anti-BGG.

Discussion. The inability of 6-MP treatment to inhibit antibody formation to BGG in the guinea pig, or to suppress skin sensitization to picryl chloride in this species, is in complete contrast to the capacity of the drug to block totally the primary humoral antibody response in rabbits. The basis for this divergence is unknown. However, the possibility that some species of animals may be inherently insusceptible to 6-MP as an inhibitor of immunological responses (perhaps because of unusual pathways for destruction or elimination of the drug) must be seriously considered. Meeker *et al.*(7) have suggested that the effect of 6-MP in prolonging skin homograft survival may be limited to certain species, since this particular effect could be demonstrated in rabbits but not in mice. On the other hand, the same laboratory group has reported without experimental details, that 6-MP prevents the production of allergic encephalomyelitis in guinea pigs(4). Even though this observation may be substantiated, the present experiments indicate clearly that the guinea pig is in a totally different class from the rabbit with respect to the effect of 6-MP.

TABLE III. Delayed-Type Sensitization to Picryl Chloride in 6-MP Treated Guinea Pigs.

6-MP treatment	No. of animals showing skin reactions* Neg.	Intermed.	Mod. strong
6 mg/kg	1	1	3
40 "	0	2	2
None	0	2	2

* Neg., Intermed., Mod. strong = practically no erythema, faint pink reaction, pale pink or greater erythema at 48 hr (reaction peak). Two normal animals (toxicity controls) gave Neg. reactions.

The gross toxicity of 6-MP for rabbits, manifested by weight loss and sometimes by death, is well known(1,5,8,9) and is seen again in the present work; in contrast, the physical condition of all our guinea pigs receiving the drug remained good. That is, 400-750 g animals treated with 40 mg/kg/day remained at about their initial weight, while younger animals (300-350 g) treated with 9 mg/kg/day actually gained weight, although not as rapidly as controls.

Summary. Administration of 6-mercaptopurine in doses as great or greater than those which effectively inhibit the immunological responses of rabbits failed to show suppressive action in guinea pigs. No depression was found in the production of precipitins by guinea pigs following mild stimulation by a soluble protein antigen (BGG), nor in development of delayed hypersensitivity in response to sensitization by a simple chemical (picryl chloride). These findings are incompatible with the view that 6-MP is a general inhibitor of antibody synthesis in all animal species.

ADDENDUM. After submission of this article for publication our attention was directed to a statement by Salvin and Smith (J. Exp. Med., 1960, v111, 465) that 6-MP in quantities up to 75 mg/kg/day does not prevent the appearance of delayed or Arthus types of hypersensitivity in guinea pigs.

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Solubilization of Serotonin and Related Compounds in Benzene-*n*-Butanol in Presence of Human Plasma.* (26802)

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Recent studies in this laboratory(1) have suggested that, in the presence of a nondialyzable factor present in human plasma, serotonin is taken up *in vitro* by human platelets as a complex with divalent cations. Uptake of serotonin by intact platelets was considerably enhanced by presence of plasma. The enhancement of uptake was partially prevented by addition of disodium EDTA or of Mg^{++} but not by addition of disodium calcium EDTA; it was blocked by simultaneous addition of disodium EDTA and Mg^{++}. Serotonin was shown further to be capable of solubilizing calcium phosphate, indicating some form of complexing with Ca^{++}.

In the present study the extent of solubilization of serotonin and related compounds in fat solvents was explored further in presence and absence of human plasma. An *in vitro* system was used, patterned after that of Woolley(2) who has extracted a lipid from animal tissues capable of solubilizing serotonin in benzene-butanol in the presence of Ca^{++}. This extract has been postulated to contain the nonenzymic portion of serotonin receptors. Woolley later demonstrated(3) increased transport of radioactive Ca^{++} into benzene-butanol *in vitro* in the combined presence of added receptor substance and serotonin.

Human plasma was found capable of solubilizing serotonin and a number of other substances in benzene-butanol. However only serotonin and tryptamine of the substances tested showed reversal of this effect in the combined presence of divalent cations and chelators. The overall findings supported the hypothesis that divalent cations bound to a portering substance or substances present in human plasma could form a complex with serotonin or tryptamine to solubilize these substances in benzene-butanol in the absence of competing chelators.

Methods. In experiments patterned after those of Woolley(2), one ml of physiologic saline (with or without added Ca^{++} or Mg^{++}, 270 μM, and/or disodium EDTA,† pH 7.4, or disodium calcium EDTA,† pH 6.9, 270 μM) was added to 6.0 ml of platelet-free plasma (from venous blood, using disodium EDTA and processing as in earlier studies (4) but discarding platelets) either nondialyzed or dialyzed (revolving against a total of 75 volumes in 5 increments of balanced

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† As Endrate Disodium or Endrate Disodium Calcium, kindly made available by Abbott Laboratories.

TABLE I. Solubilization of Serotonin in Benzene-Butanol in Presence of (a) Non-dialyzed and (b) Dialyzed Human Plasma.*

	Serotonin (0.25 μM) added to:*	Plasma added*	Serotonin ($\mu M \times 10^{-2}$) recovered from benzene-butanol	Duplicate recoveries, % variation
(a)	Balanced buffered salt solution	None	.86	2.2
	Idem + disodium EDTA	"	.67	0
	Physiologic saline	Non-dialyzed	2.45	1.5
	Disodium EDTA + saline	"	1.83	4.1
	" calcium EDTA + saline	"	2.37	3.1
	$MgSO_4$	"	1.74	1.1
	Disodium EDTA + $MgSO_4$	"	.61	7.6
	" calcium EDTA + $MgSO_4$	"	1.56	5.4
(b)	Physiologic saline	Dialyzed	.74	9.8
	Disodium EDTA + saline	"	.71	6.9
	" calcium EDTA + saline	"	.74	8.3
	$MgSO_4$	"	.81	7.5
	Disodium EDTA + $MgSO_4$	"	.81	3.1
	" calcium EDTA + $MgSO_4$	"	.89	4.1

* See *Methods* for details.

buffered salt solution‡ plus original disodium EDTA concentration of 2.69×10^{-3}M over a 48-hour period at 5°C). Control tubes with 6.0 ml of balanced buffered salt solution (pH 7.6) instead of plasma were included in every experiment. Test substance (0.25 μM) was added in 1.0 ml of balanced buffered salt solution or saline as indicated in tables, with or without added Ca^{++} or Mg^{++}, 270 μM. The mixture was shaken vigorously in glass-stoppered tubes with 10 ml benzene-*n*-butanol (1:1) for 5 minutes at room temperature and then centrifuged at 100 g to completely clear the benzene-butanol layer. Five ml of the clear top organic layer was transferred to a clean glass-stoppered tube containing 2 ml 0.1N HCl. The tube was shaken and centrifuged as before; the organic solvent was suctioned off and discarded, and 10 ml benzene were added to the aqueous layer. After re-shaking and re-centrifuging, the acid aqueous layer was transferred with a Pasteur pipette to a quartz cuvette for analysis of spectrum and measurement of test substance, using an Aminco-Bowman Spectrophotofluorometer. The test substances used, and the activation/fluorescent wave length used for measurement, were as follows: "L" Tryptophan (N.B.C.) at 270 mμ/330 mμ, tryptamine HCl (N.B.C.) at 270 mμ/355 mμ; serotonin creatinine sulfate (kindly made available by Upjohn Co.) at 295 mμ/335 mμ; 5-hydroxyindole-3-acetic acid (5HIAA; Calif. Found. Bioch. Research) at 285 mμ/335 mμ; 1-benzyl-2-methyl-5-methoxytryptamine (BAS; kindly made available by Merck, Sharp and Dohme) at 290 mμ/360 mμ; tyramine monohydrochloride (N.B.C.) at 270 mμ/330 mμ; norepinephrine (Levophed Bitartrate, Winthrop) at 270 mμ/320 mμ. A 3-point standard curve of test substance in appropriate amounts in 0.1N HCl was measured simultaneously in each instance. The nature of the experiment precluded subjecting the standards to the entire extraction procedure, since untreated standards were insoluble in benzene-*n*-butanol. However, as noted, controls of 0.25 μM of test substance in 7 ml of balanced buffered salt solution (pH 7.6) were carried through the entire procedure in each experiment so that the amount of test substance carried over non-specifically in each extraction could be assessed. All tests were run in duplicate. Experimental variation for each determination is indicated in the accompanying tables. With 3 exceptions noted in Table III, all determinations checked within 10%.

Results. Results are summarized in Tables I-III. In the presence of human plasma, sero-

‡ Ten ml stock solution (7.5% NaCl, 0.75% KCl, 0.1% Na_2HPO_4, 0.12% KH_2PO_4, 0.05% K_2HPO_4) and 7 ml 1% Na_2HPO_4 per 100 ml, aqueous (total phosphate concentration, 6.7×10^{-3}M).

TABLE II. Effect of Divalent Cations on Solubilization of Serotonin in Benzene-Butanol.*

Serotonin (0.25 μM) added to:*	Plasma added*	Serotonin ($\mu M \times 10^{-2}$) recovered from benzene-butanol	Duplicate recoveries, % variation
(a) Balanced buffered salt solution	None	.86	2.2
Physiologic saline	Non-dialyzed	2.45	1.5
$CaCl_2$	"	1.35	4.8
Disodium EDTA + $CaCl_2$	"	.70	7.8
$MgSO_4$	"	1.74	1.1
Disodium EDTA + $MgSO_4$	"	.61	7.6
(b) Physiologic saline + $CaCl_2$	None	.56	4.9
$CaCl_2$	Non-dialyzed	1.35	4.8
PO_4 buffer (0.25M) + $CaCl_2$	None	.25	7.3

* See *Methods* for details. (a) and (b) refer to comments in text (see *Results* and *Discussion*).

tonin was partially solubilized in benzene-butanol (Table I-a). In testing the effect of dialyzed plasma in the system, the original plasma concentration of added disodium EDTA was maintained in order to take advantage of the tighter chelating action of EDTA over that of the plasma proteins. Thus both originally bound and free divalent cations were dialyzed out of the plasma, to the extent achievable by use of 75 volumes of dialyzing balanced buffered salt solution with added disodium EDTA over a period of 48 hours. As noted in Table I-b, solubilization of additional serotonin into benzene-butanol in the presence of plasma was completely suppressed by prior plasma dialysis which, among other things, was sufficient to remove virtually all originally bound and free Ca^{++} and Mg^{++} from the plasma.

In the absence of competing chelator, overall solubilization of serotonin was somewhat decreased by addition of Ca^{++} or Mg^{++} in the presence of nondialyzed plasma (Table II-a). Addition of either Ca^{++} or Mg^{++} and disodium EDTA with its tighter chelating action than plasma-binding-substances completely suppressed the enhanced solubilization of serotonin in benzene-butanol in the presence of plasma (Table II-a). Addition of disodium calcium EDTA had little suppressive effect in the presence of Mg^{++} (Table I-a).

Substitution of hyperphysiologic phosphate buffer (0.25M, pH 7.0) for plasma in the *in vitro* system to which serotonin and Ca^{++} were added resulted in suppression of solubilization of serotonin in benzene-butanol (Table II-b).

As shown in Table III, serotonin was not alone among substances tested in being solubilized in benzene-butanol by a factor or factors present in human plasma. The degree of solubilization on addition of plasma was proportionally greatest with tyramine and least with the serotonin analogue, BAS. However, only serotonin and tryptamine exhibited reversal of enhanced solubilization in benzene-butanol by human plasma when disodium EDTA and Mg^{++} were added. Only tryptamine (Table III-b) of the substances tested showed a pattern of change to all test additions (*i.e.*, disodium EDTA, disodium calcium EDTA, Mg^{++} and combinations thereof) which was identical to the pattern exhibited by serotonin (Table I-a).

In the studies reported in Table III, the fluorescent spectra of the substances extracted from benzene-butanol solution into 0.1N HCl were identical with those of the original substance used, with 3 exceptions: Tyramine and norepinephrine showed a slight but definite shift of fluorescent spectrum toward the visible end of the spectrum whenever plasma had been used in the *in vitro* system. It was thought possible that the test substance in each instance might have displaced some naturally occurring substance from its plasma binding site and that this substance was then solubilized in benzene-butanol and extracted. However, this could never be established, if so, and the substances measured were definitely shown not to be

TABLE III. Solubilization of Related (to Serotonin) Compounds in Benzene-Butanol.*

Compound added (.25 μM) to:*	Plasma added*	Recovery of added compound (μM $\times$ 10^{-2}) from benzene-butanol	Duplicate recoveries, % difference
(a) *Tryptophan**			
Balanced buffered salt solution	None	1.24	0
Idem + disodium EDTA	"	1.17	5.7
Physiologic saline	Non-dialyzed	2.42	2.8
Disodium EDTA + saline	"	2.43	1.4
" calcium EDTA + saline	"	2.43	1.4
$MgSO_4$	"	2.43	1.4
Disodium EDTA + $MgSO_4$	"	2.52	0
" calcium EDTA + $MgSO_4$	"	2.45	0
(b) *Tryptamine**			
Balanced buffered salt solution	None	4.21	9.7
Idem + disodium EDTA	"	3.92	3.7
Physiologic saline	Non-dialyzed	8.35	1.4
Disodium EDTA + saline	"	6.69	1.7
" calcium EDTA + saline	"	7.49	1.6
$MgSO_4$	"	6.66	2.6
Disodium EDTA + $MgSO_4$	"	3.46	1.7
" calcium EDTA + $MgSO_4$	"	6.58	1.8
(c) *Tyramine**			
Balanced buffered salt solution	None	1.39	6.1
Idem + disodium EDTA	"	1.31	6.5
Physiologic saline	Non-dialyzed	7.69	6.6
Disodium EDTA + saline	"	7.39	19.4
" calcium EDTA + saline	"	8.79	7.7
$MgSO_4$	"	7.69	6.6
Disodium EDTA + $MgSO_4$	"	6.33	8.0
" calcium EDTA + $MgSO_4$	"	7.69	6.6
(d) *BAS**			
Balanced buffered salt solution	None	.84	8.6
Idem + disodium EDTA	"	.99	9.8
Physiologic saline	Non-dialyzed	1.34	0.9
Disodium EDTA + saline	"	1.28	1.9
" calcium EDTA + saline	"	1.12	10.8
$MgSO_4$	"	1.26	5.7
Disodium EDTA + $MgSO_4$	"	1.31	5.5
" calcium EDTA + $MgSO_4$	"	1.31	5.5
(e) *5HIAA**			
Balanced buffered salt solution	None	.15	25.0
Idem + disodium EDTA	"	.15	0
Physiologic saline	Non-dialyzed	.57	9.8
Disodium EDTA + saline	"	.56	6.7
" calcium EDTA + saline	"	.56	6.7
$MgSO_4$	"	.61	3.1
Disodium EDTA + $MgSO_4$	"	.60	0
" calcium EDTA + $MgSO_4$	"	.61	3.1
(f) *Norepinephrine**			
Balanced buffered salt solution	None	.32	8.7
Idem + disodium EDTA	"	.31	0
Physiologic saline	Non-dialyzed	1.34	2.1
Disodium EDTA + saline	"	1.22	2.2
" calcium EDTA + saline	"	1.32	4.2
$MgSO_4$	"	1.22	2.2
Disodium EDTA + $MgSO_4$	"	1.06	2.6
" calcium EDTA + $MgSO_4$	"	1.20	2.3

* See *Methods* for details.

serotonin by analysis of spectrum. 5HIAA showed a slight but definite shift of fluorescent spectrum toward the visible end of the spectrum in all samples following extraction, with or without addition of plasma to the *in vitro* system. These were consistent and reproducible observations.

Discussion. Enhanced solubilization of serotonin in benzene-butanol in presence of human plasma was suppressed when virtually all originally-bound and free divalent cations were dialyzed out of the plasma prior to testing (Table I-a). When Ca^{++} or Mg^{++} was added in molecular excess in the presence of plasma, however, solubilization was somewhat decreased below the level attained with nondialyzed plasma alone (Table II-a). If solubilization of serotonin in benzene-butanol depended simply on complexing with free divalent cations, unchanged or increased solubilization might have been expected in the circumstances, and presence of plasma should not have been necessary. Such not being the case, the observations suggested that plasma bound divalent cations were important in the solubilization and that addition of an excess of free Ca^{++} or Mg^{++} caused a shift in equilibrium of complexing of serotonin with bound and free divalent cations.

The complete suppression of the solubilization of serotonin seen in the presence of plasma in benzene-butanol on addition of either Ca^{++} or Mg^{++} and disodium EDTA to the *in vitro* system (Table II-a) was expected from earlier data, in view of the tighter chelating action of disodium EDTA over that of plasma binding substances. The order of addition of reactants to the system, prior to extracting with benzene-butanol, placed serotonin last in the system. Thus serotonin apparently complexed with divalent cations bound either to plasma components or to chelator. No fat solubilization could be demonstrated with EDTA-chelated complexes, however. Addition of disodium calcium EDTA had little suppressive effect on solubilization of serotonin benzene-butanol in the presence of Mg^{++} (Table I-a), in contrast. Since the molar concentration of chelated magnesium far exceeded the molar concentration of either calcium or serotonin, this suggested preferential (and perhaps sole) binding of serotonin with Ca^{++} rather than by Mg^{++}. Where suppression of fat solubilization of serotonin was seen in the presence of excess Mg^{++} and disodium EDTA, it is probable that the higher stability constant of Ca^{++} chelates over Mg^{++} chelates resulted in considerable transfer of bound calcium from plasma components to the tighter binding of the added chelator before the added Mg^{++} was chelated.

Under the conditions of the present study, while solubilization of several substances in benzene-butanol was demonstrable in the presence of plasma (Table III), only tryptamine of the substances tested shared with serotonin the characteristics which suggested a possible role of plasma bound calcium in its solubilization in benzene-butanol. Collier(5) has summarized fairly recently many biologic actions shared by tryptamine and serotonin. Whether the present findings will ever be shown to be related to biologic events in the sense of indicating a role in the transport of bound calcium through lipoidal cellular walls is a matter of conjecture at the moment. It is of interest that the *in vitro* conditions imposed in the present studies duplicated those of certain of the platelet uptake studies reported earlier(1). Where this was true, some parallelism was evident between degree of solubilization of serotonin in benzene-butanol in the present studies and degree of uptake of serotonin by intact platelets in the earlier studies. A chief difference between the present and former studies where *in vitro* conditions were otherwise duplicated was in the use of 75% platelet-free plasma in the present experiments rather than platelets in 4% plasma as in the prior(1) experiments. The effect of varying plasma concentration has not yet been tested in the present system, nor has plasma fractionation been done as yet to try to determine wherein plasma activity lies as regards solubilization of the test substances in benzene-butanol. Both studies are planned.

Summary. Using an *in vitro* system patterned after that of Woolley(2), serotonin, tryptamine, tyramine and several related test substances were solubilized in benzene-bu-

tanol in varying degree in the presence of human plasma. Only serotonin and tryptamine of the substances tested were significantly influenced in their fat solubilization in the presence of plasma by addition of an excess of Ca^{++} or Mg^{++} and/or chelators to the *in vitro* system. Solubilization of both serotonin and tryptamine in benzene-butanol in presence of plasma was decreased somewhat by addition of Ca^{++} or Mg^{++} or disodium EDTA in excess to the system. It was eliminated by addition of disodium EDTA (but not disodium calcium EDTA) and either Ca^{++} or Mg^{++} to the system, also by prolonged prior dialysis of the plasma against balanced buffered salt solution plus disodium EDTA. The overall findings supported the hypothesis that divalent cations bound to a substance or substances present in human plasma formed a complex with serotonin or tryptamine to solubilize these usually lipid-insoluble materials in benzene-butanol, in the absence of competing chelators.

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Influence of Epinephrine and Fasting on Free Fatty Acid Mobilization in Goldthioglucose-Induced Obesity. (26803)

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Regulatory and metabolic types of obesity in mice have been described by Mayer(1). In the former, accelerated rates of lipogenesis are considered to be a consequence of hyperphagia resulting from hypothalamic damage. In the latter, it is suggested that hyperphagia is secondary to hyperlipogenesis arising from an inherent metabolic defect.

Recently it was reported that obese-hyperglycemic mice (O-H), representing metabolic obesity, exhibit an impairment in their ability to mobilize free fatty acids (FFA) *in vitro*, from adipose tissue in response to fasting and epinephrine stimulation(2).

To determine whether the defect is related to the etiology of the obese-hyperglycemic syndrome, or if it is a nonspecific consequence of obesity *per se*, the influence of fasting and epinephrine stimulation was investigated in goldthioglucose† obese (GTG) mice, an example of regulatory obesity.

Materials and methods. 50-60 mg portions of epididymal adipose tissue were obtained from goldthioglucose-induced obese CBA mice in the dynamic phase of obesity and from lean controls. Obese mice were approximately 5 months old, weighed 42-50 g, had been obese for 2 months, and were still actively gaining weight. Lean mice were the same age and weighed 27-37 g. Tissues were incubated in a Krebs-Ringer bicarbonate medium containing 0.1% glucose and 2% bovine serum albumin. FFA content of adipose tissue and release into the medium were measured 3 hours following addition of either epinephrine* or, as in the case of controls, water. Analytical methods used are identical with those reported earlier(2).

Results and discussion. Adipose tissue from fed goldthioglucose obese mice and lean controls produced equivalent amounts of FFA during incubation without hormonal stimulation (Fig. 1). In the lean group, epinephrine induced an increase in FFA content of both tissue and medium which amounted to a 6-fold increase in total FFA production ($p<.001$). Similarly, epinephrine

* Adrenalin, Parke Davis Co., Detroit, Mich.

† Goldthioglucose, courtesy Schering Corp., Bloomfield, N. J.

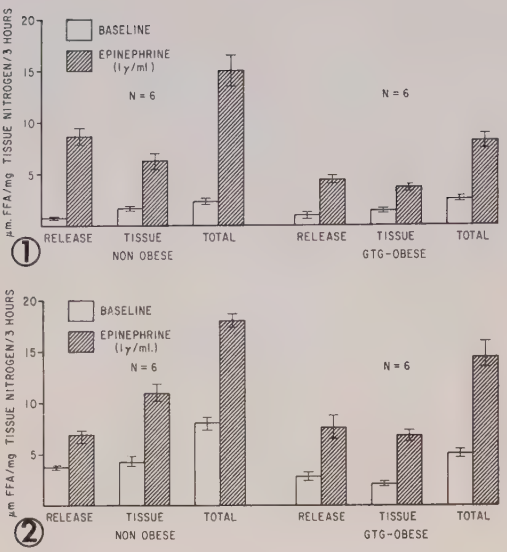

FIG. 1. The influence of epinephrine on epididymal adipose tissue content and release of FFA in fed goldthioglucose induced obese mice during dynamic phase of obesity and in lean CBA mice.

FIG. 2. The influence of epinephrine on epididymal adipose tissue content and release of FFA in fasted goldthioglucose induced obese mice during dynamic phase of obesity and in lean CBA mice.

stimulation in the obese group resulted in a 3-fold increase in total FFA production ($p < .001$).

Results obtained with 16-18 hr fasted animals are represented in Fig. 2. Baseline FFA production in adipose tissue of lean and obese mice was increased significantly ($p < .001$) in response to fasting; the response of the former, however, was greater than the latter. In both groups, epinephrine evoked an equivalent increase in FFA mobilization ($p < .001$).

These results reveal that adipose tissue from goldthioglucose obese mice, unlike that of O-H mice(2), significantly increases FFA production in response to fasting and epinephrine stimulation. Moreover, the response of tissue obtained from fasted GTG mice was of similar magnitude as that of tissue from lean animals. An impaired response was, however, observed in tissue from fed GTG obese mice; also, tissues from lean mice gave a greater response to fasting than did tissue from obese mice.

Accelerated rates of lipogenesis have been reported for fed and fasted obese-hyperglycemic mice and for fed (but not fasted) goldthioglucose obese mice(3). Since tissues from fed and fasted O-H mice and fed GTG obese mice also show an impairment in FFA mobilization, it is suspected that the diminished response to fasting and epinephrine stimulation may be in part a reflection of hyperlipogenesis.

FFA concentrations of adipose tissue and incubation medium are influenced by 2 processes: a) rate of lipolysis of tissue triglyceride and b) rate of esterification of FFA(4). Esterification of FFA by adipose tissue has been shown to be dependent upon the nutritional state of the animal in that adipose tissue from fasted rats incorporates less FFA into triglyceride than does tissue from fed animals (5). It is possible that FFA released from triglyceride in response to epinephrine stimulation are more rapidly re-esterified in tissues actively synthesizing lipid (*viz.*, fed and fasted O-H and fed GTG mice), thus suggesting an apparent diminished mobilization of FFA. Accelerated rates of re-esterification of FFA, therefore, may account for the diminished response of adipose tissue from fed GTG mice to the lipolytic influence of epinephrine, and may also be a component in the impaired mobilization of FFA in adipose tissue from O-H mice.

Summary. Epididymal fat pad adipose tissue obtained from goldthioglucose obese mice mobilized free fatty acids (FFA) when incubated with epinephrine and in response to a 16-hr fast. FFA production was similar in adipose tissues of fasted lean and obese mice; however, the response of adipose tissues of fed obese mice was less than that of fed lean animals. It is suggested that rate of re-esterification of FFA may be a factor in the apparent mobilization of FFA in adipose tissue.

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Effects of Valine-5 Angiotensin II on Excretion of Water and Salt in Primary and Secondary Hypertension.* (26804)

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Intravenous administration of angiotensin in hypertensive subjects promotes an increased excretion of water and salt(1). In contrast, a similar infusion in normal subjects usually results in sodium retention and antidiuresis(2). Whether angiotensin causes natriuresis in subjects with hypertension by acting directly on the kidney, or by some other mechanism is not clear. Since hypertensives seem to ingest more salt than normotensives (3), it may well be that this dietary factor conditions their response to angiotensin.

The present study deals with the effects of intravenous angiotensin under conditions of high and low salt intake on renal excretion of sodium, potassium and water in subjects with hypertension due to primary aldosteronism, advanced essential hypertension and chronic glomerulonephritis.

Material and methods. Six subjects were studied. One (H.M.) had primary aldosteronism, 2 (H.F., O.J.) advanced essential hypertension, and 3 (S.J., B.E., R.D.) chronic glomerulonephritis. Resting diastolic blood pressure ranged between 110 and 130 mm Hg in H.M., H.F. and O.J., and from 90 to 100 mm Hg in S.J., B.E. and R.D. Degree of renal disease varied from severe (S.J., B.E., R.D., H.F.) to moderate (O.J.) or no impairment (H.M.). None of the subjects presented symptoms or signs of congestive heart failure.

All subjects were maintained on a fixed Na (30 to 90 mEq/day) and K (50 to 90 mEq/day) intake for one to 6 weeks. Daily urines were measured and samples of the individual diets saved. After establishing Na retention (urinary Na 20 to 80 mEq/day below intake for at least 10 days) in H.M., H.F. and O.J., and Na depletion (urinary Na exceeding intake by 20 to 30 mEq/day for 2 weeks) in S.J., B.E. and R.D. an intravenous infusion of 300 cc of 5% D/W was given over 6 to 24 hours. On the following day, 100 to 200 μg of synthetic valine-5 angiotensin II (angiotensin II)† diluted in 300 cc 5% D/W was administered intravenously during 6 to 24 hours.

In one of the subjects (R.D.) on each of the following 2 days the effects of intravenous aldosterone‡ alone, 0.5 mg diluted in 300 cc 5% D/W, and in combination with angiotensin II were also studied.

Observations similar to the above were repeated in the subject with primary aldosteronism (H.M.) after removal of an adrenal cortical adenoma, in one subject (O.J.) with advanced hypertension after 3 weeks of reduced Na intake (20 mEq/day), and in 2 subjects (B.E., R.D.) with chronic glomerulonephritis after Na depletion had been corrected by provision of additional salt in tablet form (65 and 100 mEq Na/day).

All subjects were kept recumbent throughout the infusions. Blood pressure was determined at 5 to 30 minute intervals during administration of angiotensin II, and less frequently during infusion of 5% D/W. Blood was drawn before and at termination of infusions.

Pooled diet samples and urines were analyzed for sodium and potassium by flame

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† Hypertensin Ciba, lot E6429
‡ d-aldosterone Ciba, lot E6403.

TABLE I. Effects of I.V. Angiotensin II on Urinary Excretory Function in Primary Aldosteronism and Essential Hypertension. Values of urine volume (V), sodium (U_{Na}), potassium (U_K), osmolality (U_{Osm}), urine sodium to potassium ratio (U_{Na}/U_K), endogenous creatinine clearance (C_{Cr}) and blood pressure (BP).

Subject	Intake (meq/day)	Reg.	V (l/day)	U_{Na} (meq/l)	$U_{Na} \times V$ (meq/day)	U_K (meq/l)	$U_K \times V$ (meq/day)	U_{Na}/U_K	U_{Osm} (mOsm/l)	C_{Cr} (ml/min.)	BP (mm Hg)	Angiotensin (μg/min.)
H.M.					Before adrenalectomy							
	Na 50	C	1.40	18	25	37	52	.48	333	81.0	190/120	
	K 65	Angiot.	2.70	49	134	30	82	1.64	280	96.0	246/155	.4
		R	1.74	23	41	29	50	.82	286	92.0	196/100	
H.F.	Na 90	C	.89	12	11	54	48	.23	423	11.0	215/124	
	K 60	Angiot.	1.00	28	38	56	56	.68	408	10.0	248/136	.4
O.J.	Na 40	C	1.23	17	21	45	56	.37	584	46.2	220/120	
	K 90	Angiot.	1.91	39	74	40	77	.96	476	46.3	240/130	.4
H.M.					After adrenalectomy							
	Na 60	C	1.60	76	121	32	52	2.32	416	90.0	180/110	
	K 75	Angiot.	1.60	55	88	42	67	1.51	419	91.5	214/130	.5
O.J.	Na 20	C	1.91	22	43	32	62	.70	303	49.7	220/117	
	K 90	Angiot.	1.92	4	8	28	55	.14	320	49.7	230/122	.4

Reg. = regimen; C = control; Angiot. = angiotensin II; R = recovery.

TABLE II. Effects of I.V. Angiotensin II on Urinary Excretory Function in Chronic Glomerulonephritis. Values of urine volume (V), sodium (U_{Na}), potassium (U_K), osmolality (U_{Osm}), urine sodium to potassium ratio (U_{Na}/U_K), endogenous creatinine clearance (C_{Cr}) and blood pressure (BP).

Subject	Intake (meq/day)	Reg.	V (l/day)	U_{Na} (meq/l)	$U_{Na} \times V$ (meq/day)	U_K (meq/l)	$U_K \times V$ (meq/day)	U_{Na}/U_K	U_{Osm} (mOsm/l)	C_{Cr} (ml/min.)	BP (mm Hg)	Angiotensin (μg/min.)
S.J.	Na 30	C	1.60	38	61	32	51	1.20	115	16.0	140/90	
	K 50	Angiot.	1.50	34	51	41	61	.83	118	16.0	150/98	.4
B.E.	Na 60	C	2.10	38	80	19	39	2.05	275	18.0	135/90	
	K 80	Angiot.	1.60	42	67	40	64	1.05	333	18.0	142/95	.6
R.D.	Na 30	C	2.00	29	58	15	30	1.93	194	7.0	162/80	
	K 80	Angiot.	1.95	29	56	15	29	1.93	186	7.2	194/100	.2
		d-aldost.	1.96	15	30	23	45	.67	186	7.6	167/88	
		Angiot. + d-aldost.	1.79	18	33	24	43	.77	187	6.5	197/100	.3
B.E.	Na 160	C	1.43	55	78	38	55	1.42	377	19.0	140/85	
	K 80	Angiot.	1.70	66	112	32	53	2.12	354	19.0	150/92	.6
R.D.	Na 95	C	1.34	36	48	24	33	1.45	208	6.1	180/90	
	K 80	Angiot.	1.41	43	61	25	35	1.74	227	6.3	215/105	.3

Reg. = regimen; C = control; Angiot. = angiotensin II; d-aldost. = d-aldosterone 0.5 mg.

photometry. Plasma and urine creatinine chromogen concentration was measured by the method of Bossnes and Taussky(4), and urine osmolality by the Fiske osmometer.

Results. In Tables I and II are listed the observed data relative to blood pressure (BP), 24-hour urine volume (V), urine sodium (U_{Na}), potassium (U_K), osmolality (U_{Osm}), urinary sodium to potassium ratio ($U_{Na/K}$) and endogenous creatinine clearance (C_{Cr}).

Effects of angiotensin II infusion in primary aldosteronism and advanced hypertension (Table I). 1. Administration of angiotensin II under conditions of Na retention promoted in H.M. (before adrenalectomy), H.F. and O.J. a mild to marked diuresis, a more than 2-fold increase in U_{Na}/L and a 3- to 5-fold rise in U_{Na}/day. In contrast, U_K/L did not change, while U_K/day increased from 12 to 16% and $U_{Na/K}$ more than 50% above control. C_{Cr} rose by 18% in H.M., but did not change in H.F. and O.J.

2. After Na depletion had been established in H.M. (3 weeks following adrenalectomy) and O.J. angiotensin II infusion did not alter V, though U_{Na}/L and U_{Na}/day decreased 18 and 33 mEq, respectively. U_K/L and U_K/day changed little, consequently $U_{Na/K}$ decreased markedly. C_{Cr} did not change in either subject.

Effects of angiotensin II infusion in chronic glomerulonephritis (Table II). 1. Administration of angiotensin II under conditions of Na depletion promoted a slight to moderate decrease of V in S.J. and B.E., but not in R.D. U_{Na}/L remained nearly unchanged in all the 3 subjects, while U_{Na}/day decreased 10 and 13 mEq in S.J. and B.E. U_K/L and U_K/day tended to rise in S.J. and B.E., while it did not change in R.D. $U_{Na/K}$ diminished by 10 to 50%, and C_{Cr} remained unchanged.

The infusion of aldosterone in R.D. caused a fall in U_{Na}/L and U_{Na}/day of about 50%, and a small rise in U_K/L and U_K/day. Consequently, $U_{Na/K}$ decreased markedly. When angiotensin II was added to the aldosterone infusion on the following day, no further decrease of U_{Na} or increase of U_K were found, though V and C_{Cr} fell by 8.7 and 14.6% respectively.

2. After a state of Na retention had been established in B.E. and R.D. administration of angiotensin II caused a slight to moderate rise in V, U_{Na}/L and U_{Na}/day. Since U_K/L and U_K/day were not affected by the infusion, $U_{Na/K}$ increased. C_{Cr} did not change in either subject.

Discussion. These observations show that under conditions of sodium retention angiotensin promotes natriuresis and diuresis in hypertensive subjects. By contrast, under conditions of sodium depletion angiotensin tends to cause a diminished excretion of salt and water. The results are in accord with observations made in the rat(5,6), and emphasize the role of sodium metabolism in renal response to angiotensin.

The fact that angiotensin caused exaggerated natriuresis in the 2 subjects with advanced hypertension (H.F., O.J.) as well as in those with chronic glomerulonephritis (S.J., B.E., R.D.) and in that with primary aldosteronism (H.M.) indicates that the phenomenon is not a specific feature of essential hypertension. Peart and Brown(7) have found that angiotensin-induced natriuresis could be abolished in hypertensives by lowering the blood pressure to normal levels. It is pertinent in this respect that 3 subjects of the present study (H.M., H.F., O.J.) having a diastolic blood pressure above 110 mm Hg exhibited the greatest natriuresis during infusion of angiotensin. Thus, the results suggest that renal response to angiotensin is related to the height of blood pressure and is conditioned by the state of sodium balance.

The mechanism of angiotensin-induced natriuresis in hypertensive subjects is obscure. Understanding of the phenomenon is further complicated by the complexity and variety of factors affecting the renal handling of sodium. Renin causes less renal vasoconstriction in the hypertensive than in the normotensive dog(8), and angiotensin in hypertensive subjects does not significantly affect glomerular filtration(7). In the patient with primary aldosteronism (H.M.) the natriuretic response to angiotensin before adrenalectomy was associated with a rise of endogenous creatinine clearance of 18%. This finding suggests that the natriuresis in this sub-

ject might have been due to an increased filtered sodium. However, there were no changes in endogenous creatinine clearance in the other subjects exhibiting natriuresis (H.F., O.J.), nor in those in whom sodium excretion decreased or did not change. One might speculate that angiotensin acts directly on the renal tubule causing a diminished reabsorption of sodium. However, this hypothesis is difficult to reconcile with the fact that adrenal secretion of aldosterone is elevated in hypertensives and is further increased by administration of angiotensin(2, 9). Another hypothesis might be that the natriuresis induced by angiotensin represents the effect described in the dog(10), wherein a rise in the kidney perfusion pressure causes increased sodium excretion without changing the glomerular filtration rate.

Summary. Angiotensin II was administered at rates of 0.3 to 0.6 μg per minute over 6 to 24 hours to 6 subjects with hypertension of various etiologies under conditions of positive and negative sodium balance. Angiotensin produced hypernatriuresis under condition of positive sodium balance. The effect was most marked in those subjects having a diastolic blood pressure above 110 mm Hg.

Miss S. Goodman, Mrs. S. Beacher and Miss M. E. Novotny provided technical assistance. Angiotensin II (Hypertensin Ciba) and aldosterone (d-aldosterone Ciba) were obtained through the courtesy of Dr. W. E. Wagner, Summit, N. J.

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Ovarian Weight Response to Varying Doses of Estrogens in Intact and Hypophysectomized Rats.* (26805)

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A dose-response relationship was demonstrated for injected diethylstilbestrol and ovarian weight stimulation in hypophysectomized immature rats after a 4-day treatment interval(1). A recent study has shown that the greatest increase in ovarian weight occurs after about 8 days of treatment with large doses of estrogen in both intact and hypophysectomized immature rats(2). The following experiments were carired out to evaluate ovarian weight response after 8 days of injection with varying doses of diethylstilbestrol or estradiol.

Materials and methods. Intact or hypophysectomized Sprague-Dawley rats, 23-25 days old, were utilized in groups of 5 animals each. Hypophysectomized rats were received on the second post-operative day from Hormone Assay Laboratories, Chicago, Ill. Cortisone acetate (2.5 mg) was administered upon arrival and estrogen injections were begun on the following day. Daily injections of 4, 20, 100, 500, or 1000 μg of either diethylstilbestrol or estradiol, dissolved in 0.1 cc peanut oil, were given for 8 days. Uninjected control animals were caged separately. The animals were asphyxiated with natural gas 24 hours after final injection. The ovaries were dissected free, weighed on a Roller-Smith torsion balance, and prepared for microscopic examination.

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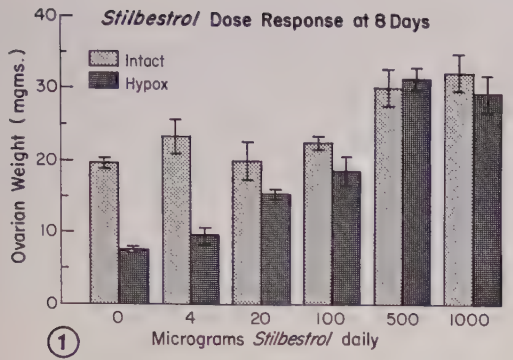

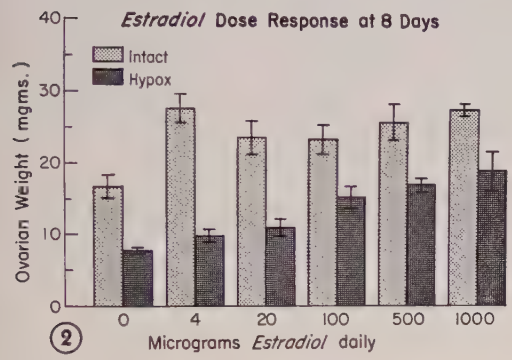

FIG. 1. Avg ovarian weights of rats treated with varying doses of diethylstilbestrol for 8 days. Stand. error of mean indicated by vertical bars.

FIG. 2. Avg ovarian weights of rats treated with varying doses of estradiol for 8 days. Stand. error of mean indicated by vertical bars.

Relative follicle size was estimated by examination of randomly selected fields in several ovarian cross-sections. An eyepiece grid was utilized which outlined an area of 1 sq mm at 100 power magnification. All follicles within this area were counted and the average number of follicles per grid area was determined for each dose level of stilbestrol.

Results. Fig. 1 depicts average ovarian weights of rats treated with varying doses of diethylstilbestrol. Step-wise increases in ovarian weight were produced in hypophysectomized rats with increasing doses of estrogen. The greatest response was observed with 500 μg, although this did not differ significantly from the average weight attained with 1000 μg doses.

No clear-cut sequential pattern of ovarian weight stimulation was detected in the intact animals subjected to doses of 100 μg or less. However, the 2 largest doses brought about significant increases which were comparable to ovarian weights observed in hypophysectomized animals treated in the same manner.

Average ovarian weight response to estradiol injection is illustrated in Fig. 2. Slight step-wise increases were produced in hypophysectomized rats and the sequence was similar to that obtained with diethylstilbestrol. However, the weights attained after treatment with the larger doses of estradiol were not as great as those produced by comparable doses of stilbestrol.

In intact rats, all the test doses of estradiol increased ovarian weight over control level; but no definitive dose-response pattern was apparent. The smallest and largest doses were equally effective.

Histologically, ovarian response to injected estrogen was similar to that reported previously. Many solid follicles were present, distributed uniformly throughout the ovary and some of the larger follicles showed the peculiar peripheral degeneration that others have described(1,2,3,4).

The averages of several counts of the number of follicles present within a 1 sq mm area of the ovaries from each test group of hypophysectomized rats are listed in Table I. Since the fields examined consisted only of follicles with a very slight amount of interstitial tissue intervening, one could assume that the average follicular cross-sectional area approached the reciprocal of the number of follicles counted. By estimating follicle size in this manner, it appears that the 20 and 100 μg doses produced comparable increases; and 500 and 1000 μg produced another similar increment (Fig. 3). No counts were done on the untreated control ovaries since the interstitial component between follicles occupied

TABLE I. Follicle Population per sq mm Ovarian Cross-Section after Diethylstilbestrol Treatment. Hypophysectomized rats.

Daily dose (μg)	Mean No. of follicles/mm^2
4	30.2
20	20.8
100	19.2
500	11.3
1000	11.7

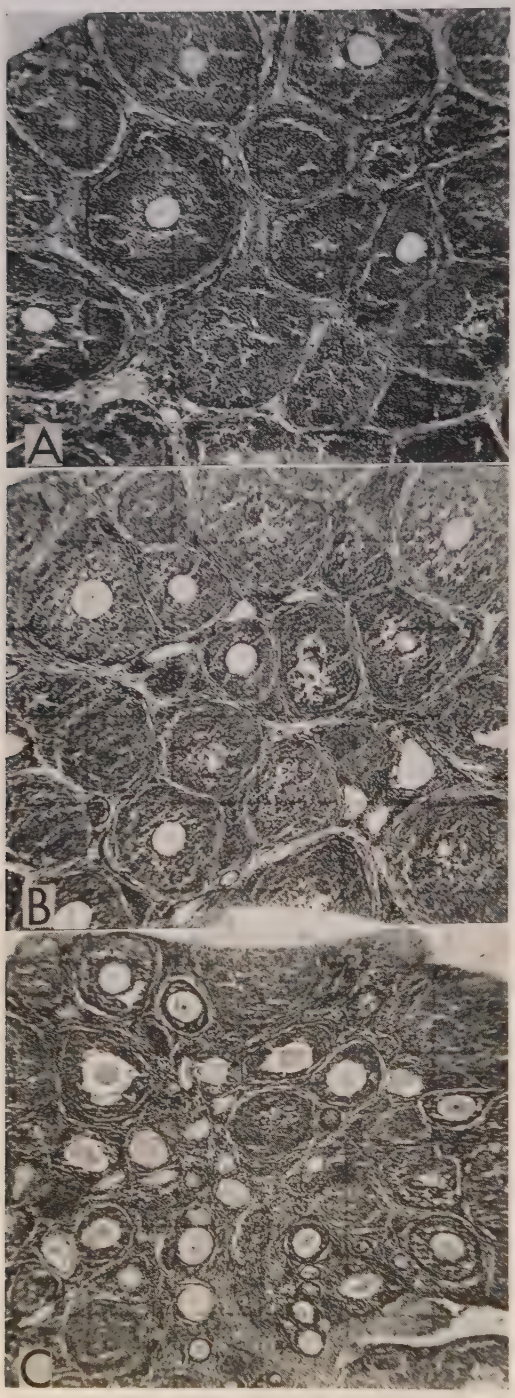

FIG. 3. Photomicrographs of ovaries from hypophysectomized immature rats.

(A) 8 days treatment with 1000 μg stilbestrol daily.

(B) 8 days treatment with 4 μg stilbestrol daily.

(C) Untreated control.

an inordinate amount of the grid area.

Discussion. The dose-response relationship observed after 8 days of stilbestrol treatment was similar to that previously reported for 4 days of treatment(1). However, after 8 days treatment, higher average ovarian weights were noted with the large doses in hypophysectomized rats. Also, as has been previously suggested, stilbestrol is more effective in stimulating ovarian growth than is the natural estrogen, estradiol(1,4).

The presence of the pituitary body undoubtedly accounts for the lack of a dose-response relationship in intact rats. Bradbury reported that small doses of estrogen can cause release of gonadotrophins by the pituitary of immature rats, and thereby indirectly increase ovarian weight(5). However, it is felt that the resulting enhancement of ovarian growth after injection of large doses of estrogen in both intact and hypophysectomized rats is due to a direct estrogen stimulation of the ovary. This proposed direct effect is further documented by the results in hypophysectomized animals where a dose-response relationship is manifest.

Williams found a graded ovarian response to increasing doses of estrone (up to 200 μg), although ovarian weights did not exceed 11 mg(4). He proposed that the ovarian growth was due to granulosal proliferation, resulting in greater follicle size. Other investigators have shown that small doses of estrogen (less than 1 μg) cause slight increases in follicle size(6). The microscopic findings of the present study support the observation that ovarian response to exogenous estrogen is granulosal proliferation. This granulosal response is apparently directly proportional to the dose of estrogen administered, as documented by increasing follicle size. Enhanced follicular growth is in turn reflected in the ovarian weights which rise as the estrogen dosage is increased.

Summary. A dose-response relationship was demonstrated between ovarian weight and injected estrogen in hypophysectomized immature rats. No definitive pattern of response was evident in intact animals treated similarly. In hypophysectomized animals, increases in follicular size were produced as

estrogen dosage was raised. The follicular growth was characterized by granulosal proliferation, resulting in many solid follicles. Apparently, ovarian weight increments resulting from increasing estrogen doses are reflections of this enhanced follicular growth.

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Competition of Antigens as Influenced by Spacing of Heterologous Antigen Injections.* (26806)

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Decreased responses to some antigens following injection of 2 or more antigens have been observed by a number of investigators (1-5). In some cases a crowding out has been shown to occur when an antigen is injected into an animal actively undergoing antibody formation against a previously injected antigen(6-9). Benjamin and Witzinger(7) found that a preliminary injection of horse serum inhibited the formation of hemolysin to sheep cells. Glenny *et al.*(8) found a crowding out of the response to diphtheria toxoid in guinea pigs when the toxoid was injected simultaneously with, or 2, 4, or 6 days after, an injection of normal horse serum. Frolova *et al.*(9) observed a reduction in precipitin response of guinea pigs to ox serum injected 7-14 days after sensitization with horse serum.

In some cases a more rapid response to one antigen in a mixture has been postulated as leading to a crowding out of antibody production against other antigens in the inoculum(1-3). Adler(2) found that simultaneous injection of rabbit serum or hemocyanin with rabbit immune globulin into guinea pigs impeded antibody production against the rabbit immune globulin and that the degree of this suppression was related to the intensity of antibody response to the competing antigens. Abramoff and Wolfe(3), in quantitative studies of the primary response to bovine serum albumin (BSA) and human gamma globulin (HGG), at a 20 mg per kilogram body weight (mg antigen/KBW) dosage level, observed that the chicken system responds earlier to HGG than it does to BSA and the HGG is thus able to crowd out the response to BSA when these antigens are simultaneously injected. However, Abramoff(10) has shown that the selectivity of HGG could be reduced during simultaneous injection of BSA and HGG, at the 40 mg/KBW dosage level, if the animals had been presensitized to BSA. Under these conditions the responses to both of these antigens were significantly reduced.

The present investigation is concerned with further clarification of the mechanisms by which one antigen interferes with antibody production against a second antigen during primary antibody responses. Responses of chickens to single, simultaneous, and spaced injections of BSA and HGG at a dosage level of 40 mg/KBW have been studied.

Materials and methods. Eighty-two New Hampshire Red Chickens (74 males, 8 females) of approximately 34 weeks of age and weighing 2300-4000 g were used in these

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TABLE I. Precipitin Production in Chickens* Injected with 40 mg per kg Body Weight of Bovine Serum Albumin (BSA) and/or Human γ-globulin (HGG).

Days after antigenic stimulus	Group I Single inj. BSA (14)‡	Group II Single inj. HGG (15)	Group III Simultaneous inj. BSA and HGG (15)		Group IV HGG inj. 24 hr after BSA (20)		Group V HGG inj. 48 hr after BSA (19)	
	Antibody (in μg) nitrogen/ml of undiluted serum†							
	BSA	HGG	BSA	HGG	BSA	HGG§	BSA	HGG§
3	0	0	0	0	0	0	0	0
4	0	+	0	+	0	0	0	0
5	+++	133 ± 23	+	171 ± 24	+++	0	42 ± 20	0
6	126 ± 26	280 ± 34	47 ± 18	421 ± 66	82 ± 17	80 ± 10	197 ± 54	0
7	326 ± 52	298 ± 58	328 ± 40	350 ± 60	151 ± 19	200 ± 25	400 ± 55	99 ± 25
8	369 ± 55	245 ± 55	441 ± 59	239 ± 38	172 ± 18	219 ± 22	371 ± 53	291 ± 47
9	314 ± 45	191 ± 50	402 ± 62	190 ± 37	175 ± 16	166 ± 20	281 ± 29	334 ± 45
10	227 ± 36	134 ± 34	358 ± 53	155 ± 36	166 ± 19	110 ± 14	228 ± 21	271 ± 47
12	191 ± 32	78 ± 24	245 ± 46	107 ± 28	113 ± 18	66 ± 10	161 ± 21	210 ± 46
14	138 ± 25	52 ± 19	120 ± 36	68 ± 24	75 ± 14	23 ± 6	94 ± 18	105 ± 23
16	59 ± 12	20 ± 8	75 ± 23	39 ± 18	47 ± 14	12 ± 5	35 ± 13	62 ± 14
18	25 ± 8	12 ± 7	38 ± 13	21 ± 11	11 ± 11	+++	18 ± 8	27 ± 7

* Animals 34 wk of age when inj.
† Mean ± stand. error.
‡ Number in parentheses represents No. of animals in each group.
§ Antibody response to HGG measured from inj. of BSA.

experiments. Antigenic stimulation consisted of intravenous injections of purified soluble BSA‡ and/or HGG§ prepared in 1% saline and standardized to contain 40 mg protein per ml. All injections were given on the basis of 40 mg/KBW.

The chickens were treated as follows: Group I: 14 birds received a single injection of BSA;‖ Group II: 15 birds received a single dose of HGG;‖ Group III: 14 animals received successive injections of BSA and HGG within a period of 30 seconds; Group IV: 20 chickens received an HGG injection 24 hours following BSA stimulation; Group V: 19 birds received an HGG inoculation 48 hours after a BSA injection.

Blood was obtained by wing vein or cardiac puncture begining the second or third day after first injection and subsequently on the 4th, 5th, 6th, 7th, 8th, 9th, 10th, 12th, 14th, 16th, and 18th days. The sera were stored at −20°C previous to testing for antibody nitrogen. Individual serum samples were assayed for antibody nitrogen using the Heidelberger quantitative precipitin technic as previously described(10).

Results. Antibody responses to simultaneous injections of BSA and HGG. Earlier studies(3) had shown that antibody responses of animals simultaneously injected with 40 mg/KBW each of BSA and HGG showed equivalent decreases of approximately 50% to both of these antigens as compared with their respective controls. However, these animals were bled only on the 8th day following simultaneous stimulation and it was suspected that peak antibody responses to these 2 antigens were being reached at different times. The present study, in which the complete curve of antibody response was analyzed following simultaneous injection of 40 mg/KBW, showed that antibody responses to BSA and HGG did indeed reach their peaks on different days (Fig. 1B and Table I). It can also readily be seen that antibody responses to BSA and HGG did not differ significantly from their respective controls. These results are in contrast to earlier studies at the 20 mg/KBW dosage level(3). At this dosage level antibody response to BSA was significantly reduced in mean peak titer and occurrence of maximum antibody titer was delayed from the 8th day following a single injection of BSA to the 10th day following simultaneous injection with HGG.

‡ Pentex, Inc., Kankakee, Ill.

§ Generously supplied by J. H. Hink, Cutter Laboratories, Berkeley, Calif.

‖ Injection and bleeding of animals used in this series of experiments were carried out during the same period of time as injection and bleeding of animals used in a study previously reported(10). Therefore, Groups I and II serve as controls for both of these studies.

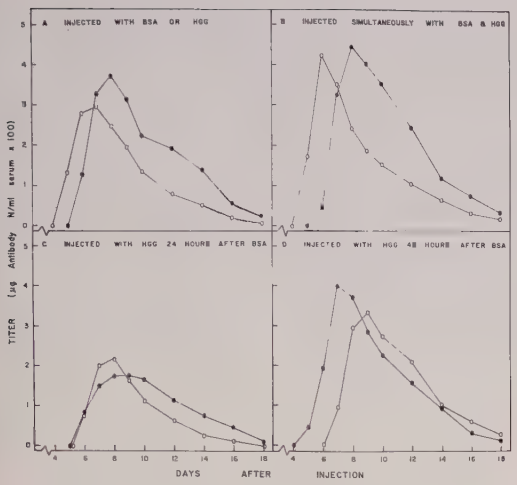

FIG. 1. Precipitin production curves of chickens following single injections (A) of bovine serum albumin (BSA)(●) or human gamma globulin (HGG)(○), or a combination of these 2 antigens inj. simultaneously (B) or at 24 (C) or 48 (D) hr intervals. Curves C and D plotted from day of BSA inj.

The antibody response to HGG was unaffected.

Antibody responses to BSA and HGG following a 24 hour delayed injection of HGG. Earlier studies(3) also had shown a 2-day delay in appearance of the peak response to BSA following simultaneous injection of 20 mg/KBW each of BSA and HGG. Furthermore, it had been demonstrated that at 40 mg/KBW the selective advantage of HGG over BSA could be partially overridden by simultaneous inoculation of BSA and HGG into animals previously sensitized to BSA (10). Consequently, in the present study, injection of HGG was delayed to determine whether this might provide an additional means of influencing the ability of HGG to alter the response to BSA at a dosage level of 40 mg/KBW.

Results of the experiment in which the injection of HGG was withheld until 24 hours after that of BSA indicate that the response to BSA was significantly reduced (Table I, Fig. 1A and 1C). Thus, although the response to HGG was itself somewhat reduced, these results indicate that, at this dosage level, such a delay of HGG injection enables HGG to regain the selective advantage over BSA previously observed at the 20 mg/KBW dosage level.

Antibody responses to BSA and HGG following a 48 hour delayed injection of HGG. Results of the preceding experiment raise the question as to whether a lapse of 48 hours between injection of BSA and HGG would produce an even greater suppression of the response to BSA. Whereas antibody response to BSA was reduced following a 24-hour delayed injection of HGG, it can readily be seen that antibody responses to BSA and HGG in animals in which the injection of HGG was delayed for 48 hours approximate the responses of their respective controls (Table I, Fig. 1A and 1D). Statistical analyses in which responses of Groups II and V to HGG were treated as if superimposed in time, revealed no significant differences between them. Similarly, the amount of antibody produced against BSA in this experiment was not significantly different from that of its control group.

Discussion. It has been shown previously that both bovine serum albumin and human gamma globulin are "good" antigens in the chicken system(3,10). However, HGG has been shown to possess certain selective advantages over BSA(3). At the 20 mg/KBW level chickens which readily produced satisfactory amounts of antibody in response to single injections of BSA produced significantly less antibody against this antigen when concurrently injected with HGG. Furthermore, the peak response to BSA was delayed 2 days, whereas the response to HGG remained unchanged. In the present studies the selectivity of HGG was altered under circumstances of simultaneous injection of BSA and HGG at the 40 mg/KBW level insofar as antibody response to HGG failed to suppress the response to BSA. These data suggest that specific optimal dosage levels exist for each antigen which permit expression of its maximal antibody response. In this respect, some correlation with the secondary response studies of Barr and Llewellyn-Jones (4) is possible. They found that a preliminary injection of *H. pertussis* vaccine brought about interference with the diphtheria response following subsequent immunization

with a combined alum-precipitated diphtheria-pertussis prophylactic. Interference did not occur, however, when the dosage of both antigens in the secondary injection was increased considerably.

It is also evident that the ability of one antigen to interfere with the antibody response to another is dependent upon the prior immune state of the host(1,4,10,11). Abramoff(10) has shown that the selectivity of HGG can be reduced during simultaneous injection of BSA and HGG, at the 40 mg/KBW level, if the animals are presensitized to BSA. The present studies, at the same antigenic concentration, indicate that the selectivity of HGG over BSA may also be reduced by delaying injection of HGG for 48 hours.

The mechanism by which spacing of injections influences the ability of one antigen to alter the antibody response to another antigen appears to be associated with altered induction periods. A competition occurred between BSA and HGG under those experimental conditions in which induction periods were altered so as to be of the same duration for both antibody responses; *i.e.*, simultaneous injection at 20 mg/KBW of each antigen (3), simultaneous injection of 40 mg/KBW of each antigen following previous sensitization with BSA(10), and HGG injection 24 hours following BSA stimulation (present study). Thus, it would appear that under conditions in which these 2 antigens are predisposed to stimulate the antibody-forming mechanism at approximately the same time, a competition of antigens occurs, the extent of which then appears to be conditioned by dosage(3 and present study), spacing of injections, and previous immune state of the animals(10).

Summary. 1) Antibody responses of chickens to single, simultaneous, and spaced injections of bovine serum albumin and human gamma globulin at a dosage level of 40 mg per kilo of body weight are described. 2) Under conditions of simultaneous injection of BSA and HGG antibody responses to both BSA and HGG approximate those of their respective controls. 3) Results of the experiment in which injection of HGG was withheld until 24 hours after that of BSA indicate that antibody response to BSA was significantly reduced. 4) Antibody responses to BSA and HGG in animals in which injection of HGG was delayed for 48 hours approximate the responses of their respective controls. 5) It appears that the character of the antibody response to simultaneous injections of BSA and HGG is regulated by the parameters of dosage level and timing of injections.

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Effect of Chlorpromazine upon Calcification *in vitro*. Interaction with Isolated Epiphyseal Cartilage of Rachitic Rats.* (26807)

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It has been shown that a turbidimetric interaction occurs between chondroitin sulfate (CSA) and chlorpromazine solutions(1). Since CSA is the main component of cartilaginous tissues, and is considered to play a role in the calcification process(2-5), it was considered of interest to determine whether chlorpromazine would interfere with the ossification of epiphyseal cartilage. Therefore, a study was carried out to determine the effects of exposure of epiphyseal cartilage obtained from rachitic rats to various concentrations of chlorpromazine solution, with reference to calcification *in vitro*.

Material and methods. Calcification *in vitro* was studied by simplification of the method described by Sobel, Nobel and Hanok(6). Twenty-one day old weanling rats were raised on U.S.P. Rachitogenic Diet No. 2. Three weeks later they were sacrificed, and the tibia was dissected and sliced longitudinally. The tibial sections were placed in calcifying solution and incubated at 37°C for 20 hours. To observe the presence or absence of calcification, the sections were washed with water, placed in 2% aqueous silver nitrate and exposed to the light of an incandescent lamp for about 10 minutes. Calcification appeared as a dark deposit on the surface of the epiphyseal plate. The extent of this deposit was estimated by using numbers from 0 to 4 for the width of plate covered, and by 0, or 1 to 4 plus signs for the length of plate covered. Using this method the range of values can extend from 0 (0) to 4 (++++). To obtain a figure representing overall calcification for graphic purposes, the plus signs representing length were treated as numbers (0 to 4), and multiplied by the figure representing width. This could give a theoretical maximum value of 4 (4+) = 16.

The calcifying solution used in these experiments contained sodium chloride (0.07M), potassium chloride (0.005M), sodium bicarbonate (0.022M), calcium chloride (0.0025M; 10 mg % calcium) and a combination of NaH_2PO_4 plus Na_2HPO_4 at a molarity of 0.00161 corresponding to 5 mg % phosphorus. The pH was adjusted to 7.3 with CO_2.

Luteocobalti chloride was prepared according to the method of Fernelius(7). All other chemicals used in this study were of reagent grade.†

Measurements of turbidity were made with the Bausch and Lomb Spectronic-20 spectrophotometer, set at a wavelength of 420 mu. Measurements of metachromasy were made with the same instrument, using as an index of metachromasy an Optical Density Ratio (ODR) which is equal to OD 550/OD 635. pH measurements were made with the Beckman Model G, pH meter.

Results. Exposure of tibial sections to various concentrations of chlorpromazine resulted in deposition of the compound, as a white precipitate, over the surface of the epiphyseal plate.

Exposure of tibial sections to a solution containing 1.0% chlorpromazine and 5% luteocobalti chloride (pH 5.81) for 1½ hours, was not followed by the usual deposition of cobalt salt, indicating interference by chlorpromazine. Normally both rachitic and non-ossifiable cartilage would bind luteocobalti chloride(3), forming an orange deposit.

Treatment of tibial sections for one hour with chlorpromazine (*i.e.*, 0.5% or 1.41×10^{-2}M) followed by shaking in a solution of toluidine blue O plus calcium chloride (*i.e.*,

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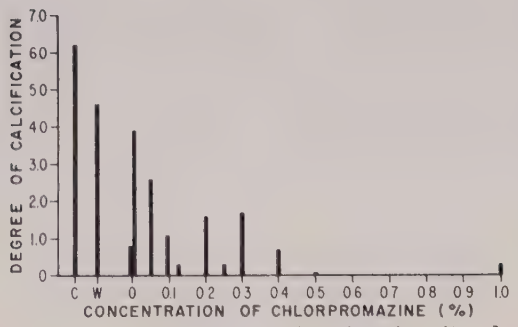

FIG. 1. Inhibition of calcification *in vitro* by prior exposure of sections to chlorpromazine. Sections exposed to concentration of chlorpromazine (as indicated) for one hr, then placed in calcifying solution for 20 hr at 37°C. "C" represents control (N = 22), "W" represents water (N = 18). Total No. of treated sections was 51.

1.06×10^{-5}M of dye and 0.125 N of calcium chloride), was not followed by metachromasy. Usually rachitic cartilage will stain metachromatically with solutions of toluidine blue O and calcium chloride. Concomitant exposure to both 0.5% chlorpromazine and the toluidine blue O calcium chloride solution, not only inhibited metachromasy, but caused the deposit of chlorpromazine to become more clearly visible on the epiphyseal plate.

When chlorpromazine was incorporated in the calcifying solution at the pH required for calcification *in vitro* (*i.e.*, 7.3) the process of calcification was inhibited. Shortly after mixing chlorpromazine with the calcifying solution, a turbidimetric interaction occurred, then at the end of incubation period required for calcification, a deposition of needle-like crystals was noticed. To eliminate a possible direct effect of chlorpromazine upon the calcifying solution, sections of rachitic cartilage were exposed to chlorpromazine prior to incubation. The sections were shaken for one hour and then washed with water and incubated in the calcifying solutions for 20 hours at 37°C.

The results (Fig. 1) while manifesting some degree of random variation, indicate that calcification is inhibited by concentrations of chlorpromazine as low as 0.1% (2.8×10^{-3}M). Further, degree of calcification appeared inversely related to concentration of chlorpromazine.

Untreated control sections gave an average degree of calcification of 6.2. A total of 27 treated sections were also used as controls to the chlorpromazine experiment. Eighteen sections were treated with distilled water, and 9 were shaken with various concentrations of HCl down to pH 4.3. In both cases degree of calcification did not go below 4.0. In the case of unacidified distilled water, average degree of calcification for the 18 sections was 4.6.

Inhibition of calcification *in vitro* by other agents, *i.e.*, protamine(2,8) and luteocobalti chloride(3) was found to depend on the ratio:

$$\left(\frac{\text{Calcium}}{\text{Inhibitory cation}} \right)$$

meaning that addition of calcium to the solution promoted calcification, preventing inhibition by the inhibitory cations. This reversal of the effect of the inhibitor was also produced by calcium in the case of chlorpromazine as shown in Table I.

Fig. 2 shows that the turbidimetric interaction between chlorpromazine and CSA is a rate phenomenon, the velocity of which steadily decreases over the 35 minute period of study. In the presence of calcium ion (Fig. 2, curve B) rate of growth is initially increased, and a maximum optical density of 1.20 is reached in 10 minutes. From this point curve B descends slowly, while curve A (*i.e.*, without calcium ion) continues to rise, so that by the end of the experiment the 2 curves show tendency to intersect.

When 1.0 N HCl was progressively added to the chlorpromazine-CSA system, no significant change in turbidity occurred until the solution reached pH 2 (Fig. 3). As pH decreased below this point the solution became pink, losing 72% of its turbidity by the time it reached pH 1.0. The pink color was similar to that of chlorpromazine solutions that have been extensively exposed to light.

The chlorpromazine-CSA system was also found to be sensitive to temperature (Fig. 4). With increase in temperature (BC) there was a decrease in turbidity, so that at 70°C the turbidity has been reduced by 88%. Upon recooling the system, turbidity rose again, this time to a higher value than before (CD).

TABLE I. Reversal of Chlorpromazine Inhibition of Calcification *In Vitro* by .5 N $CaCl_2$.

	Sample	Time of exposure, hr	Exp. 1 No. of exp.	Exp. 1 Avg calcification	Exp. 2 No. of exp.	Exp. 2 Avg calcification
A	Control	—	5	4.0	4	3.9
B	Water	1	4	1.8	4	2.6
C	CPZ (1%)	1	4	.5	5	.3
D	"C" followed by .5 N $CaCl_2$	2	4	4.6	5	5.6

Significant changes in turbidity started from a temperature of about 25°C and above.

When chlorpromazine was added to the toluidine blue O-CSA system (*i.e.*, 1.06 × 10^{-5}M toluidine blue-O plus 200 γ of CSA in a total volume of 20 ml) a marked loss of metachromasy was noted. At 25°C and a pH of 6.2, addition of 2 mg % chlorpromazine diminished the metachromasy by 61%.

Discussion. The present study indicates that chlorpromazine interacts with epiphyseal rachitic cartilage, depositing as a white precipitate on the plate surface, preventing binding of luteocobalti chloride, inhibiting metachromasy and finally inhibiting calcification *in vitro,* the latter process being reversed by excess calcium ions. The metachromasy of a solution of toluidine blue O and chondroitin sulfate is also suppressed on titration with chlorpromazine. Further, the interaction between chlorpromazine and CSA has been shown to be a rate phenomenon accelerated by calcium ion. This interaction is relatively stable to acid pH (down to pH 2.0) but is markedly sensitive to temperature changes beyond 25°C. In view of the partial reversibility of the inhibition of calcification *in vitro* by 0.5 N $CaCl_2$, of the demonstrated interaction between CSA and chlorpromazine, and of the competition between chlorpromazine, luteocobalti chloride and toluidine blue O for the binding site on the epiphyseal surface, it is presumed that this binding site is chondroitin sulfate, or the CSA-collagen complex of the cartilage.

Some workers consider that collagen is the essential component of the matrix responsible for nucleation and crystal growth(9), indeed Meyer has suggested that the mucopolysaccharides act as templates for development of

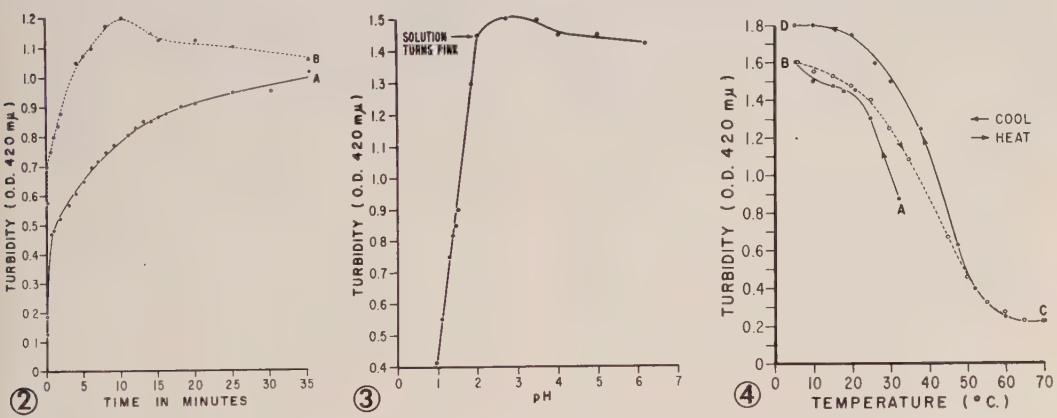

FIG. 2. Turbidimetric interaction of chlorpromazine with chondroitin sulfate as a function of time at room temperature (28°). A) 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of water. pH 6.2. B) 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of 0.1 N $CaCl_2$. pH 6.0.

FIG. 3. Effect of pH change (acid region) on interaction between chlorpromazine and chondroitin sulfate. 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml water, titrated with 1.0 N HCl at 25°C.

FIG. 4. Effect of temperature change on interaction between chondroitin sulfate and chlorpromazine. 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of water. pH 6.2.

the organized fibrous components of connective tissue(10). Nevertheless, there is a considerable body of evidence indicating that CSA or a CSA-collagen complex functions as the nucleation center. Thus, sulfated mucopolysaccharides exist at sites of both normal and abnormal calcification(11). Rubin and Howard have found an increase in metachromatic staining in regions where active calcification is occurring, which they attribute to an increase in concentration and/or a change in degree of polymerization of CSA(5). Neuman has shown that there is a correlation between the cation binding capacity of cartilage and its sulfate content(4). Miller, Waldman and McLean(12) found that basic dyes inhibit calcification *in vitro* and that this effect can be reversed in presence of calcium and phosphate. Finally it has been demonstrated that protamine(2,8) and luteocobalti chloride(3), both of which precipitate CSA from solution, can cause reversible inhibition of calcification *in vitro*.

The fact that calcification *in vitro* can be accomplished after heating rachitic sections, followed by treatment with calcium chloride, would indicate that enzyme involvement is a minimal factor in the process(11), and that chlorpromazine is not functioning as an enzyme inhibitor in the present instance. It is to be noted, however, that chlorpromazine does function as an inhibitor of a number of enzyme systems. Thus, it suppresses oxidative phosphorylation(13), cytochrome oxidase(13), ATPase(13) and D-amino acid oxidase(14).

This interference with the physical process of calcification will have more pertinent implications for the pharmacology of chlorpromazine, if it can be demonstrated that injection of the drug into the living animal, under both normal and rachitic diets, can either prevent bone formation or potentiate the rachitic state.

Summary. Sections of epiphyseal cartilage from rachitic rats, exposed to solutions of chlorpromazine in water, indicated binding of chlorpromazine to the cartilage surface. In contrast with control, the chlorpromazine pretreated cartilage showed little or no calcification after incubation for 20 hours in a calcifying solution, did not stain metachromatically with a toluidine blue O-calcium chloride solution, and did not bind luteocobalti chloride. Binding of chlorpromazine and/or calcium appeared to be of a competitive nature, excess of one reversing or preventing fixation of the other. Parallel chemical studies support the assumption that chlorpromazine interacts with the chondroitin sulfate of ossifying cartilage.

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Inhibitory Effect of Heated Vaccinia Virus on Growth of Vaccinia Virus in Earle's L Cells.* (26808)

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When cultures of L cells were inoculated with vaccinia virus at several different multiplicities (M), the yield of virus particles per culture cell varied in an inverse manner with multiplicity in the range M = 50 to M = 400(1). In order to learn whether this was caused by an excessive number of infectious particles(2) or to inhibitory effects of noninfectious virus in the inocula(3,4,5,6) experiments were done, and reported here, to test the latter possibility. Cultures of L cells have been inoculated with fresh virus preparations to which have been added known numbers of heat-treated virus particles. Other cultures have been treated with heated virus and later seeded with a challenge dose of active virus. Multiplicities and yields have been determined precisely by counting the virus particles in the electron microscope.

The observed inhibitory effects of the heated virus preparations are of such magnitude as to offer partial, if not complete, explanations of the reciprocal relationship cited above. They also introduce doubt regarding the validity of some titration methods for detecting residual active virus in heat-treated and possibly in otherwise degraded virus preparations.

Materials and methods. The vaccinia strain WR (mouse neurotropic) was obtained from the American Type Culture Collection and adapted to growth(1), in L strain cells obtained from Dr. Earle. Virus was kept in continuous passage in the cells with transfer every 48 or 72 hours. Inoculum was prepared by subjecting infected cells to 3-minute treatment with 9 kc waves.

Growth medium and details of the L cell culture have been previously described(1). Cells were scraped from the screw-capped tubes or prescription bottles in which they were grown and their number determined by means of a counting chamber. Virus particles were diluted with PBS and counted by the agar sedimentation method(7) in the electron microscope. For inoculum at a given multiplicity the virus and cells were mixed, in growth medium, in proportions calculated from the counts. These mixtures were sedimented immediately to bring virus and cells together in somewhat less than 7 minutes time(8). Heated virus was prepared by treating active virus in one ml quantities in screw-capped tubes immersed in a 56°C water bath for 45 minutes.

Just prior to inoculation all virus suspensions were treated 1½ minutes with 9 kc waves to disperse aggregates that form with standing. After sedimentation inoculation in the several centrifuge tubes, the supernatant fluid was decanted, the cells were then pooled, washed and replicate cultures set up in screw-capped tubes, each containing 10^6 cells in 1 ml of growth medium. After various intervals of incubation at 37°C tubes were removed and placed at –20°C to arrest virus growth. At the end of the experiment all tubes were thawed. The cells were scraped off, sonic treated to release and disperse the virus which was then counted in the electron microscope.

The term "active virus" will mean fresh, unheated inoculum.

Experiments and results. Freshly prepared seed virus was heat treated and applied to L cells at a multiplicity of 10. One hour later the same cells received active virus at the same multiplicity. Control cells received active virus at M = 10 at the same time. All cultures were incubated together and the virus particle development can be seen in the graph, Fig. 1. Active virus increased to nearly 9000 particles per cell at 95 hours incubation. The same virus at the same multiplicity in cells pretreated with 10 M heated virus reached a maximum production of only

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† Postdoctoral Trainee on Training Grant from U.S.P.H.S.

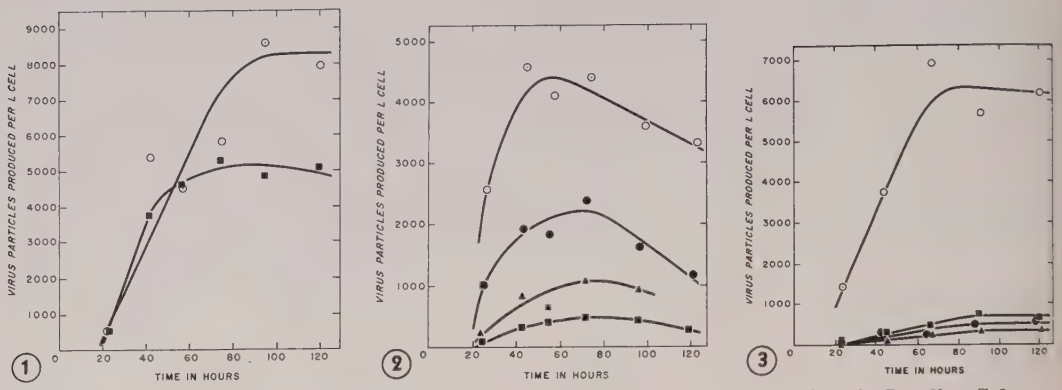

FIG. 1. Inhibitory effect of heated vaccinia virus on growth of active virus in L cells. Cultures of upper curve received active virus alone, M = 10. Those of the lower curve received M = 10 heated virus followed, 1 hr later, by M = 10 active virus.

FIG. 2. Inhibitory effect of (M = 100) heated vaccinia virus on growth of active virus applied to L cells with several time delay periods. –○–○– Control with active virus only, M = 10. –●–●– No delay (heated and active mixed). –▲–▲– 1 hr delay. –■–■– 2 hr delay.

FIG. 3. Inhibitory effect of (M = 100) heated vaccinia virus on growth of active virus applied to L cells with longer time delay periods. –○–○– Control with active virus only, M = 10. –■–■– 2 hr delay. –●–●– 4½ hr delay. –▲–▲– 21½ hr delay.

5000 particles in 72 hours which did not increase on continued incubation to 120 hours. Pretreatment of L cells with 10 M heat-treated virus 1 hour before inoculation with active virus reduced the yield by almost half.

A second experiment of the same kind but with M = 100 heated virus was performed. Active virus multiplicity was 10, the same as in the first experiment. In one set of cultures it was applied together with the heated virus. In a second set there was a 1-hour delay and in a third set a 2-hour delay between application of heated and active virus. The 6 control cultures receiving 10 M active virus produced a curve (Fig. 2) reaching a maximum yield of 4400 virus particles per cell at about 54 hours. Heated virus at M = 100 applied with the active virus reached a maximum of 2200, about ½ of the first set, in 72 hours with the 1- and 2-hour delay cultures producing 1000 and 500 particles per cell respectively. The mechanics of inoculation are such that only 4 sets of 6 cultures can be handled at once, so investigation of longer delay times had to be done in a separate experiment. For this, fresh virus and cells were used and maximum of growth in the control cultures (6200 virus particles per cell) was reached at about 72 hours, Fig. 3. The yield with the cultures having 2-hour delay time was 700 per cell or 11% of maximum, the same as the result of the first experiment. Two other sets of cultures challenged with 10 M active virus after 4½ and 21½ hours delay reached peaks of yield of 500 and 330 particles per cell respectively.

Cultures inoculated with M = 100 heated virus alone showed no virus increase during 91 hours of incubation. There was, however, some growth of residual virus which reached 172 particles per cell at 115 hours incubation. Clearly, the heat treatment was not sufficient to sterilize the preparation but the amount of remaining activity was negligible compared with the challenge dose of 10 virus particles per cell.

To check the possibility that a very small inoculum of active virus followed by a second at 10 M with a 2-hour delay would produce a growth curve different from the control, such a test was made. The initial inoculum was 0.1 M. Maximum yield of both cultures was the same within counting error, $\sigma = 16\%$.

These results, Fig. 1, reveal the appreciable inhibitory effect exerted by 10 heat-treated virus particles per L cell upon the growth of an active, M = 10 inoculum applied 2 hours later. Greater dosage of heated virus, M = 100, (Fig. 2), has reduced the growth of the challenge virus to ½ when applied with it, to about ¼ when applied 1 hour before and to

about 1/10 when applied 2 hours before. In the separate continuation experiment, Fig. 3, the depression in yield for the 2-hour delay time was again about 90% and further small changes were observed when the delay time was increased to 4½ and to 22 hours. Apparently most of the inhibitory effect of this dosage is manifest at 4½ hours.

Examination of the infected cells in the electron microscope by the methods described previously(9) showed that all cells were grossly infected in control cultures. In cultures subject to inhibitor again all cells were infected but fewer virus particles could be seen per cell. This is not the type of observation that can be quantitated but it was clear that among the cells with 100 M and 2-hour delay time (Fig. 2) there was not one cell per 10 producing virus at maximum rate but rather all cells producing virus at a reduced rate.

Each point plotted in Fig. 1-3 was obtained from the average count from 5 electron micrographs. A total of 300 pictures was made and all were counted and used.

Discussion. These are the first data, as far as the authors are aware, which show the inhibitory effect of a preparation containing a known number of heat-treated vaccinia virus particles on a known number of tissue culture cells. Although the results are characteristic of interference as described for ultraviolet-treated virus(3,4) and by others for other virus-cell systems, the possibility that a toxic reaction is involved when 100 virus particles are put upon each cell cannot be overlooked. It is true that a relatively small number, (10 particles) does produce an appreciable inhibition. It is equally apparent that 100 virus particles per cell is a large enough dose to come within the range which is often regarded as toxic. Speculation on this point is probably less profitable than consideration of this inhibitory effect upon tests for residual virus in samples treated with heat and other inactivating agents.

In these experiments maximum virus particle yield was depressed as much as 94%. These findings clearly illustrate the difficulty involved in titrating a small residual virus activity in a heated preparation such as might be used for vaccine production. The adverse effect of heated virus upon susceptible L cells has been shown. Tests have shown that this inhibition is not caused by the minute amount of active virus remaining in the heated preparation. Consequently it is necessary to reexamine titration methods designed to detect and measure residual active virus in preparations which have been heat treated or possibly inactivated by other means. A preponderance of heated virus which reduces the ability of the test cells to produce virus will be expected to alter the end point in tissue culture titrations of either LD_{50} or plaque procedures although perhaps not to the same extent. It is probable that, in such titrations, one is not learning simply the number of active virus particles but rather some subtle ratio of active to inhibiting particles; a ratio that may be quite different from one cell type to another and which would be expected to change if the inhibitor effect is itself heat labile. These remarks are not intended to cast doubt upon all titrations but only upon those in which there is opportunity for a preponderance of non-infective but inhibitory virus to predispose the test cells to reduced response.

It should be emphasized that the challenge dose of active inoculum employed in these tests, M = 10, is sufficient to incite new virus production at the rate of 130 particles per cell per hour 20 hours after centrifugal inoculation of susceptible cells. When such infected cultures have reached maximum yield all the cells can be seen, by electron microscopy, to be grossly infected. Inhibited cultures do not have even a few grossly infected cells. All seem to be producing virus but in lesser quantity.

Summary. Heated vaccinia virus has been applied to cultures of L cells followed by active virus. Multiplicities, determined by electron microscope particle count, were 10 and 100 for heated virus and 10 for challenge, active virus. Delay times ranged from 0 to 21½ hours. Substantial inhibition was observed with inoculation of heated virus at M = 10. At M = 100 the growth of a challenge inoculum applied 21½ hours later was 94% suppressed. The consequences of these

findings are discussed in connection with titration of residual virus in heat-treated virus preparations.

The authors express appreciation of the excellent technical work of S. W. Wilburn, electron microscopist.

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Biochemical Significance of Serum Glycoproteins.* I. Changes in Rat Serum Following Injury. (26809)

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A wide variety of non-specific traumatic conditions(2) are known to cause alterations in the proportions of serum proteins. Such changes in human serum have been shown by electrophoretic studies to involve a reduction in the albumin level accompanied by elevations in α_1 and α_2 globulins(2-8). In a number of diseases increased proportions of hexosamines were also observed to migrate electrophoretically as the α_1 and α_2 globulins(7). Increases have been reported in the seromucoid fraction and in total protein-bound carbohydrates(8). These observations show that the glycoproteins are important factors in the alterations of the serum proteins in the diseased state.

Changes are also known to occur in the serum proteins of laboratory animals after trauma(9-11), thus experimental injury in rats results in elevations of α_2 and β globulins(12). The total serum hexosamine level reaches a maximum value 2 to 4 days after trauma(13). According to Boas and Peterman(13) this response to injury is eliminated if the animals are kept on a starvation diet. Werner(14) prevented the elevation of total serum hexosamine in rabbits by chemically destroying the liver tissues. The elevated total serum hexosamine levels probably reflect the increased α_2 and β globulins, much as described by Bollet for various pathological conditions in humans(7). No direct observations, however, of the distribution of hexosamines following experimental injury have been reported. In this paper are described the changes in electrophoretic distribution of the hexosamine-containing glycoproteins in the serum of rats subjected to 2 standardized injuries.

Methods. Male rats (Yale, Wistar, and Holtzman strains) weighing between 200 and 300 g were injured for the "open wound" study by removing a 2.5 cm circle of skin just caudal to the scapulae (ether anesthesia) or for the "sponge" study by implanting sub-

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† Predoctoral fellow, U. S. Public Health Service. Part of the study reported here was taken from a thesis to be submitted by Mr. Chandler to the Dept. of Physiol. Chem., Wayne State Univ., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

cutaneously a 200 mg piece of Ivalon surgical sponge‡ in the same area. The 2 cm long incision was closed with a single skin clip. All of the rats were fed *ad libitum* throughout these studies. At the end of 1, 2, 4, 8 and 15 days the rats were anesthetized and a blood sample was removed by cardiac puncture. A small portion of blood was mixed with ammonium oxalate for determination of the red cell volume (hematocrit) while the remainder was allowed to clot and then centrifuged to yield the serum samples. To avoid complications due to storage, the electrophoretic studies were performed on the same day the samples of serum were obtained. Control rats were pair-fed and bled on comparable days with the experimental ones.

Samples of serum were used for determination of total protein(15) and total hexosamine(16). Proteins were separated electrophoretically§ (0.008 ml of serum) on Whatman 3 MM paper using 140 volts for 15 hours. The strips were then dried at 110°C for 30 minutes, rinsed in methanol, and stained 30 minutes in a 0.1% solution of bromphenol blue in methanol. After washing successively in 2 changes of 5% acetic acid, and once in acetate buffer (9 g sodium acetate · $3H_2O$ in 1 liter of 10% acetic acid), the strips were dried for 15 minutes at 110°C. The color was intensified by exposure to ammonia vapors and the strips were then scanned using the Analytrol automatic integrating densitometer.

For the electrophoretic distribution of hexosamine-containing glycoproteins, 0.05 ml samples of serum were applied to Whatman 3 MM paper. After electrophoretic separation was complete, the strips were dried at 110°C and stained for several minutes in the bromphenol blue solution. The heavily stained strips were washed in a 5% solution of acetic acid and then dried. Four areas were cut from the paper strips, albumin plus α_1, α_2, β, and γ globulins. The 4 portions of paper were cut into small pieces and hydrolyzed at 100°C for 6 hours in 1 ml of 2 N HCl in screw-capped test tubes. The partial hydrolysates were quantitatively filtered through Whatman No. 1 paper and applied directly to a column of Dowex 50, prepared as described by Boas(17). The filter papers and columns were thoroughly washed with water until no evidence of bromphenol blue persisted in the effluents. The hexosamines were then eluted with 2 N HCl and 5 ml of the eluates were collected in screw-capped tubes. After neutralizing these eluates (phenolphthalein) with NaOH, 1 ml of acetylacetone reagent and sufficient water were added to bring the final volume to 8 ml. The acetylacetone solution was prepared immediately before use by adding 0.6 ml of acetylacetone to 10 ml of a pH 10.2 ± 0.1 carbonate buffer (3.0 g $NaHCO_3$ and 17.2 g Na_2CO_3 in 100 ml of solution). The mixtures of sample and acetylacetone reagent were heated and extracted with isoamyl alcohol as previously described(16). To 4 ml of each of the isoamyl alcohol extracts, 1 ml of Ehrlich's reagent was added (0.6 g p-dimethylaminobenzaldehyde dissolved in 2.25 ml of concentrated HCl and 20.25 ml of isoamyl alcohol). The optical density was measured after 15 minutes in a spectrophotometer at 530 mμ. A standard curve was prepared using 3 to 40 μg of glucosamine dissolved in 7 ml of 7.25% saline.

Results. The hematocrit and total protein values were not significantly altered by the injuries inflicted (Table I). However, total serum hexosamine level (Table I), in agreement with published reports(9,13), was significantly elevated 1 to 2 days after both the open wound and sponge implantation ($P = <0.01$).

Fig. 1 shows that the relative distributions of α_2 and β globulins increased to maximum levels 2 to 4 days after injury and thereafter returned to normal. Individual variation in response was considerable. However, the concentrations (g %) of protein migrating electrophoretically as α_2 and β globulins were significantly elevated ($P = <0.01$) above control values 2 days after both injuries, (Table II). During the first 4 days the albumin decreased to a minimum for both open wound and sponge implantation studies.

‡ Polyvinyl alcohol surgical sponge. 200 mg pieces of sponge were boiled in several changes of distilled water and then were heated at 110°C for 30 min.

§ Spinco apparatus.

TABLE I. Total Serum Hexosamine and Protein Levels of Injured Rats. Forty control rats were used; 8-10 rats were used for each time period following injury. Yale, Wistar, and Holtzman rats were used throughout. No significant differences between strains were observed. All values are reported as mean ± stand. dev.

	Days after injury					
Injury	0	1	2	4	8	15
Open						
Hematocrit (%)	47 ± 4	47 ± 2	47 ± 2	46 ± 3	46 ± 2	46 ± 3
Total protein (g/100 ml)	6.6 ± .4	6.7 ± .4	6.6 ± .3	6.3 ± .2	6.3 ± .3	6.5 ± .4
Total hexosamine (mg/100 ml)	105 ±11	121 ±13	117 ±18	118 ±17	112 ±16	114 ± 9
Sponge						
Hematocrit (%)	47 ± 4	48 ± 3	43 ± 3	46 ± 3	47 ± 3	45 ± 2
Total protein (g/100 ml)	6.6 ± .4	6.1 ± 1.0	6.5 ± .7	6.3 ± .8	6.9 ± .5	6.7 ± .0
Total hexosamine (mg/100 ml)	105 ±11	113 ±12	129 ±17	112 ±14	105 ± 7	106 ±14

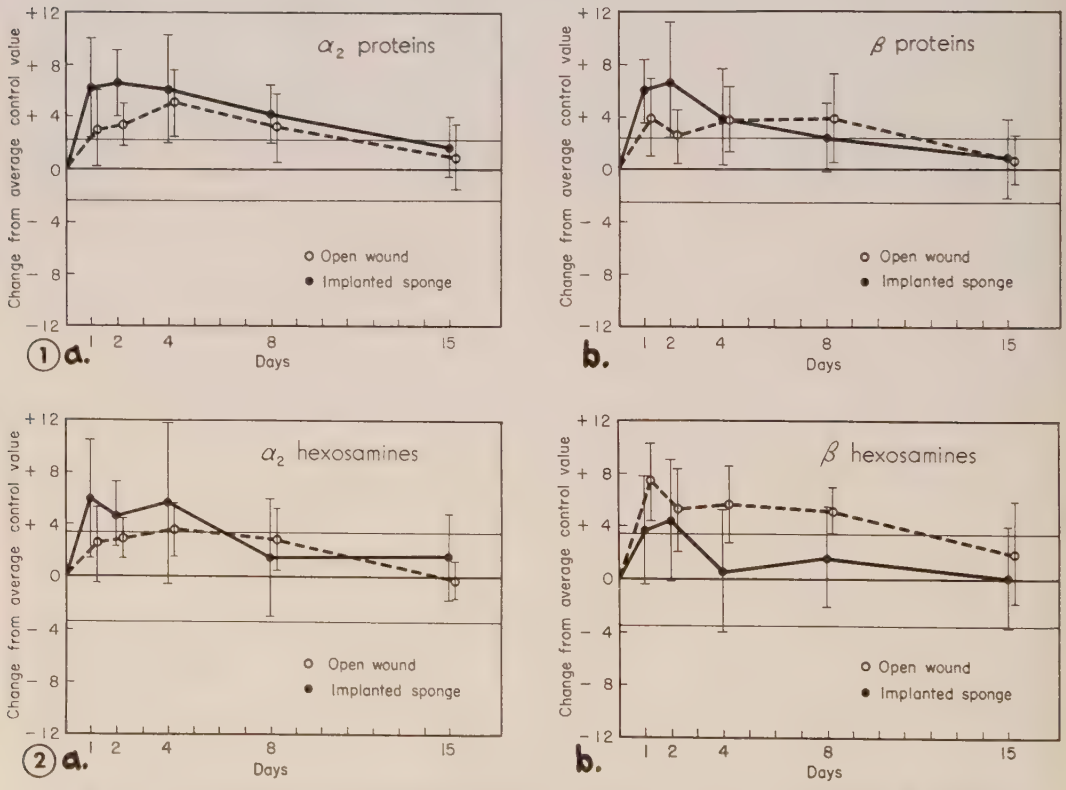

FIG. 1. Increases in serum α_2 and β globulins elicited by injury. Ordinate values represent increases above mean control value. Stand. deviations for the control are given by the lines parallel to the 0. Stand. deviations for each time period are also given. Abscissa values are days after injury.

FIG. 2. Changes in α_2 and β hexosamine-containing glycoproteins caused by injury.

TABLE II. Change in Electrophoretic Distribution of Serum Proteins and Hexosamines in Rats 2 Days Following Injury.

Injury	No. of rats	Proteins (g/100 ml)					Hexosamines (mg/100 ml)			
		Albumin	α_1	α_2	β	γ	Albumin + α_1	α_2	β	γ
None*	40	2.51 ± .34	1.15 ± .30	.83 ± .14	1.19 ± .18	.85 ± .22	56.0 ± 8.3	20.7 ± 4.0	18.6 ± 3.4	9.4 ± 2.7
Open wound	12	2.17 ± .36	1.06 ± .15	1.09 ± .13	1.36 ± .14	.94 ± .22	55.0 ± 10.0	26.0 ± 3.3	26.5 ± 6.0	9.7 ± 2.5
P†		<.05	—	<.01	<.01	—	—	<.01	<.01	—
Sponge	7	1.57 ± .29	1.24 ± .27	1.33 ± .20	1.59 ± .18	.80 ± .17	59.6 ± 11.0	30.7 ± 6.3	28.0 ± 6.5	7.0 ± 2.4
P†		<.01	—	<.01	<.01	—	—	<.01	<.01	—

* Control samples taken at 1, 2, 4, 8 and 15 days during experiment. Values did not differ and were, therefore, pooled.
† Probability.

There were no alterations in serum α_1 and γ globulin zones.

The relative distributions of α_2 and β globulins, measured as hexosamines, increased to maximum levels in 2 to 4 days (Fig. 2). Here also considerable individual variation in response was observed. At the same time the concentration of total serum hexosamine (Table I) increased to a maximum level. As a result the actual concentrations of hexosamines migrating electrophoretically as α_2 and β globulins (mg %) were significantly elevated (P = <0.01) 2 days after injury (Table II). The amount of hexosamines in the albumin + α_1 zone and the γ globulin region remained unchanged.

These data demonstrate a qualitatively parallel response to injury in the serum hexosamines and proteins which are classified electrophoretically as α_2 and β globulins. The increase in protein and hexosamine components of the α_2 globulins were of similar magnitude since the percentage of hexosamines in the protein was the same after injury as before (2.3% to 2.4% after injury compared with 2.5% in the control). In the β globulin fraction, however, the elevation of hexosamines after injury was greater than of the protein (1.8% to 2.0% after injury compared with 1.6% in the control). These results are explained by an increased production of serum glycoproteins in response to injury.

It is concluded from this study that the elevated total serum hexosamine levels elicited in rats by injury are caused by increases in the amounts of hexosamine-containing glycoproteins associated with the α_2 and β globulin fractions.

Summary. Changes produced in distribution of serum proteins of rats by injury were studied using paper electrophoresis. The electrophoretic distribution of glycoproteins was determined by measuring the concentrations of hexosamine in 4 zones, albumin + α_1, α_2, β, and γ globulins. Parallel elevations of serum hexosamines and proteins which are classified as α_2 and β globulins were observed in response to 2 kinds of experimental injury. These increases reached maximum proportions 2 to 4 days after injury.

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Nature of Polysaccharides Obtained from Endotoxins by Hydroxylaminolysis. (26810)

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Although endotoxins have been investigated extensively, the exact relationship between chemical nature and biologic reactions is still unknown. All endotoxins contain polysaccharide, lipid, certain amino compounds(1) and phosphoric acid and are known as lipopolysaccharides (LPSP). In the present paper endotoxins from 3 different species of bacteria and their lipid-free polysaccharides are subjected to a comparative study.

Materials and methods. The preparation of endotoxin has been previously described (1). The lipid moiety of the lipopolysaccharides was removed by hydroxylaminolysis (2). The separated polysaccharides were removed from the reaction mixture by centrifugation for 10 minutes at 1,700 g. The precipitates were washed once with alcohol and once with acetone and dissolved in water. The resultant solutions were dialyzed for 24 hours. After addition of 1 mg NaCl per ml the polysaccharides were precipitated with 2 volumes of acetone. The precipitates were washed with acetone and dried over sodium hydroxide under reduced pressure. The yield of polysaccharide was 50-60% on the basis of LPSP employed. Saccharides were determined by the anthrone method(3) with galactose as the standard. Amino sugars were determined by Dische's method(4) after deacetylation. The LD_{50} values(5) of endotoxins varied from 0.25 to 0.30 mg per 18-20 g mouse. The polysaccharides were non-toxic at 5 mg. Materials used in this study were those from *Neisseria gonorrhoeae* 97, *Escherichia coli* 0117:H27 and *Salmonella abortus equi*.

Results and discussion. Analytical data concerning 3 endotoxins and their polysaccharides are summarized in Table I. The nitrogen content indicates that amino compounds have been retained in the polysaccharides and the high phosphorus content points to a high degree of phosphorylation in all materials. The polysaccharides of *E. coli* endotoxin and of *S. abortus equi* endotoxin were free of lipid and in the *N. gonorrhoeae* polysaccharide the lipid content (stearin equivalent) dropped from 23.2% to 1.0%

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TABLE I. Composition of Endotoxins and Their Polysaccharides.*

No.	Type of products	N	P	Saccharide	Hexosamine	Total saccharide	Stearin equivalent
1	*E. coli* 0117:H27 LPSP	2.2	3.5	36.6	12.5	49.1	13.2
1A	Polysaccharide of same	2.3	2.4	52.3	24.2	76.5	0.0
2	*N. gonorrhoeae* 97-LPSP	2.1	3.4	22.0	20.3	42.3	23.2
2A	Polysaccharide of same	2.2	2.9	33.1	23.1	56.2	1.0
3	*S. abortus equi* LPSP	.8	2.1	47.0	7.7	54.7	11.2
3A	Polysaccharide of same	.8	2.0	60.0	4.7	64.7	0.0

* All values are given in per cent.

after hydroxylaminolysis. As expected, there was an increase in hexosamine and total saccharide content, after removal of the lipid moiety from the lipopolysaccharide molecule.

The ultracentrifuge patterns of all 3 polysaccharides reveal the presence of a major fast moving component and a minor slow moving component. The minor component is present in lowest concentration in the *N. gonorrhoeae* 97 sample. When graphically extrapolated to zero concentration of the specimens, the sedimentation constants (expressed in Svedberg units 1 S $= 10^{-13}$ cm/sec/units field) were as follows: for *N. gonorrhoeae* 97, $S_{20} = 8.3$; *E. coli* 0117:H27, $S_{20} = 9.2$ and *S. abortus equi*, $S_{20} = 9.4$. The rotor temperature was 20°C and solvent used was 0.1 M TRIS buffer, pH 8.0. Viscosity or diffusion measurements were not performed. Molecular weights were calculated from comparison of these sedimentation constants with those found for similar products and for which particle weights have been determined. Thus, the particle weight of the major component was found to be in the order of 200,000 and that of the minor component in the order of 10,000 or a small multiple thereof.

Summary. 1. There are marked differences between endotoxins prepared from 3 different species of gram negative bacteria. 2. The differences are retained after removal of the lipid component from the endotoxins by hydroxylaminolysis. Amino compounds are an integral part of both the endotoxin and polysaccharide molecules. 3. Molecular weights of the major components of the water soluble lipid-free materials are 200,000.

We wish to thank Dr. Edgar Ribi of the Rocky Mountain Laboratory for determining the molecular weights.

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Growth Inhibition of Microorganisms by Thyroid Hormones.* (26811)

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For unknown reasons thyroactive materials increase the vit. B_{12} (cyanocobalamin) requirement in rats, mice and chicks(1,2,3). This prompted the present investigation

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TABLE I. Effect of Thyroid Compounds on Growth Inhibition of *O. malhamensis*.

Thyroid compound (μg/ml)	B_{12} ($\mu\mu$g/ml)			
	No B_{12}	10	100	1000
None	.06	.6	2.1	3.0
L-Thyroxine				
.3	.06	.3	.92	2.8
1	.06	.12	.72	3.0
3,5-Diiodothyronine				
30	.06	.4	.9	2.8
100	.06	.14	.44	1.8
L-Triiodothyronine				
3	.06	.42	.96	3.0
10	.06	.14	.58	2.5
Tetraiodothyroacetic acid				
3	.06	.4	.8	1.3
6	.06	.22	.32	.32
3,5-Diiodo-L-tyrosine				
30	.06	.6	2.5	2.4
100	.06	.54	1.8	1.9

which reports some inhibitory effects of various thyroid hormones on growth of B_{12} and of non B_{12}-requiring microorganisms.

Materials and methods. The B_{12}-requirers used were (a) the mutant *Escherichia coli* 113-3, (b) *Lactobacillus leichmannii* ATCC 7830, (c) *Euglena gracilis* Z strain and (d) *Ochromonas malhamensis*. Methods for B_{12} assay with them have been described(4). The non B_{12}-requiring counterparts were: (a) *E. coli* ATCC 9637 (the parent strain of *E. coli* 113-3), (b) *L. casei* ATCC 7469, and (c) *Ochromonas danica*. Methods for growing these have been reported(5,6,7). Since all euglenids require B_{12}(8), non B_{12}-requiring controls for Euglena could not be included. Growth was recorded in optical density units by a Welch-Densichron equipped with a red-sensitive probe. The protozoa were grown at 28-32°C under light; bacteria at 37°C.

Results and discussion. Since only *O. danica* and *O. malhamensis* were affected by the thyroactive materials, detailed studies were confined to these 2 flagellates.

Ochromonas malhamensis. B_{12} overcame the growth inhibition induced by L-thyroxine, 3,5-diiodothyronine, L-triiodothyronine, tetraiodothyroacetic acid, and 3,5-diiodo-L-tyrosine (Table I); none of the sulfhydryl or other reducing compounds (Table II) or fatty acids (Table III) were active in this respect. The competitive inhibition index (ratio of inhibitor : metabolite) was approximately 300 for thyroxine, 3,000 for triiodothyronine and tetraiodothyroacetic acid, and 30,000 for 3,5-diiodothyronine. Iodinated L-tyrosine was included as a possible precursor of thyroxine; it was inert. Our findings suggest the possibility of using *O. malhamensis* as an indicator for thyrotoxic substances.

O. malhamensis has a B_{12} requirement like that of higher animals(9). When grown at the upper temperature limit its B_{12} requirement goes up steeply(10). This suggests, by analogy, that the B_{12} system in animals is strained under comparable metabolic stress (11). A B_{12} upset does exist in thyrotoxicosis(12).

Ochromonas danica. Because of the suggested interplay between B_{12} and glutathione in hyperthyroidism(13,14), some sulfhydryl compounds were included in this study. They did not counteract the inhibitory effects of the thyroid compounds on *O. malhamensis*, but *reduced* glutathione completely reversed inhibition of *O. danica* (Table II). Oxidized glutathione was not as effective as the reduced form; it did not overcome thyroxine inhibition, but was relatively effective against the other compounds. Thiouracil enhanced inhibition by all compounds. Vit. B_{12} did not reverse inhibition as it did for *O. malhamensis*. Thioglycolic acid and homocysteine overcame only diiodothyronine inhibition and cysteine overcame only the thyroxine and diiodothyronine inhibition.

Non-sulfhydryl reducing agents were also included in this study (Table II); none were as effective as *reduced* glutathione in overcoming inhibition. Ascorbic acid overcame diiodothyronine and tetraiodothyroacetic acid toxicity; it was slightly effective against thyroxine. Sodium metabisulfite was only effective against thyroxine inhibition.

Recent work on the protective action of fats on thyrotoxic rats showed that unsaturated vegetable fats promoted growth(15), an effect directly related to the linoleic acid content. Except for some reversal of triiodothyronine toxicity, linoleic, oleic, and stearic acids did not overcome the growth inhibition

TABLE II. Effect of Thyroid Hormones on Growth of *O. danica*.*

Compound (mg/ml)	Control	L-Thyroxine		3,5-Diiodothyronine		3,5,3′-Triiodothyronine		Tetraiodothyroacetic acid		3,5-Diiodo-L-tyrosine	
		30	60	100	300	10	30	10	30	100	300
No compound	1.72	1.22	.5	1.24	.88	1.56	.24	1.52	.14	1.44	.76
B_{12} (.001)	1.76	1.16	.6	1.44	.6	1.6	.36	1.68	.22	1.56	.72
Glutathione, reduced (.5)	1.8	1.88	1.92	1.82	1.78	1.88	1.84	1.72	1.72	1.7	1.58
Glutathione, oxidized (.5)	1.78	1.76	.48	1.84	1.76	1.8	1.2	1.8	.94	1.74	1.16
Thiomalic acid (.5)	1.7	1.6	1.82	1.72	1.8	1.78	1.34	1.76	.92	1.64	.68
Thiouracil (.5)	1.56	.08	.08	.1	.12	.62	.06	.08	.06	.6	.14
Thioglycollic acid (.2)	1.56	1.48	.68	1.72	1.8	1.62	.04	1.56	.32	1.52	.88
L-cysteine (.1)	1.76	1.64	1.46	1.64	1.44	1.72	.4	1.68	.34	1.42	.78
DL-Homocysteine thiolactone (.1)	1.8	1.56	.42	1.78	1.64	1.8	.42	1.76	.26	1.7	.66
Ascorbic acid (.5)	1.66	1.48	.92	1.66	1.72	1.76	.74	1.66	1.22	1.52	.34
Sodium metabisulfite (.05)	1.74	1.6	1.48	.14	.12	1.8	.3	1.0	.38	1.62	.24

* The thyroid hormones were added in concentrations indicated below for each compound in μg/ml.

of the thyroid compounds for *O. danica* (Table III); they were ineffective against inhibition of *O. malhamensis*. Polyoxyethylene sorbitan monolaurate (Tween 20), monopalmitate (Tween 40), and monooleate (Tween 80) were slightly effective; the trioleate ester (Tween 85) was completely effective in overcoming inhibition.

Animal studies have suggested that glutathione counteracts thyroxine inhibition by stabilizing endogenous vit. B_{12}(16). Lyophilized *O. danica* cells were assayed for B_{12}, but no measurable B_{12} activity was detected (the assay is sensitive to 10 $\mu\mu$g/mg of dried cells).

Mitochondrial derangement is corrected by B_{12}(16); it is postulated that B_{12} protects mitochondrial integrity by maintaining the sulfhydryl level. The absence of measurable B_{12} in *O. danica* may mean that the organism is more dependent on exogenous or endo-

TABLE III. Effect of Fatty Acids on *O. danica* Inhibition by Thyroid Hormones.

Thyroactive compounds (μg/ml)		Compounds (μg/ml)			
		No compound	Oleic, linoleic, linolenic, stearic acids (10)*	Tween 20, Tween 40, Tween 80* (50)	Tween 85 (50)
None		1.78	1.74	1.54	1.5
L-Thyroxine	40	.54	.64	1.14	1.66
	80	.22	.32	.84	1.68
3,5-Diiodothyronine	150	.54	.58	1.16	1.72
	300	.38	.22	.54	1.18
3,5,3′-Triiodothyronine	15	.84	1.2	1.26	1.76
	30	.14	.28	.5	1.58
Tetraiodothyroacetic acid	15	1.2	1.42	1.28	1.7
	30	.36	.4	.52	1.44
3,5-Diiodo-L-tyrosine	150	1.24	1.4	1.28	1.42
	300	.6	.66	.76	1.02

* Data given were obtained with the first compound of each group. Results with other compounds were similar.

genous sulfhydryl for mitochondrial protection.

O. danica resembles thyrotoxic animals in responding to fatty acids(15). Although linoleic acid was not active, Tween 85 permitted growth. The activity of Tween 85, contrasted with the inactivity of oleate and linoleate, raises the question of how much of the activity of Tween 85 is attributable to compounds other than the pure fatty acids used here. Since glutathione affects redox enzymes, the action of Tween 85 and glutathione may relate to their ability to maintain a redox potential favorable for mitochondrial function. Whether this involves the redox behavior of B_{12} itself remains to be seen. In Tetrahymena, a similar reversal of alkoxy-2,6-diaminopyrimidine inhibition has been shown for soy bean lecithin(17); the active components of the lecithin were not identified. The authors suggest that the lack of specificity of the lipid effect may mean that the inhibitors studies act as uncoupling agents. Such a possibility would apply with special force to thyroactive compounds(18).

Summary. The growth inhibition induced by thyroactive compounds on vit. B_{12} and non B_{12}-requiring microorganisms was studied. Thyroactive compounds were inactive for the B_{12}-requiring mutant *Escherichia coli* 113-3, *Lactobacillus leichmannii,* and *Euglena gracilis,* and for their non B_{12}-requiring counterparts. Only *Ochromonas malhamensis,* a B_{12}-requirer, and *Ochromonas danica,* a non B_{12}-requirer, were inhibited. Vit. B_{12} overcame the antagonistic action of the thyroid hormones for *O. malhamensis,* but not for *O. danica* where reduced glutathione and Tween 85 overcame the growth inhibition.

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Evidence for Immunization of F_1 Hybrid Mice Against Parental Transplantation Antigens. (26812)

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Generally implicit in transplantation experiments with mouse tissues has been the assumption that grafts of parental origin are nonantigenic to F_1 hybrid recipients(1-3).

* Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

This assumption, however, has been criticized on theoretical grounds(4-7) and appears to be at variance with certain experimental findings(8-12). This report presents preliminary results which are considered highly significant in that they support the belief that F_1 hybrid mice may be actively immunized against parental transplantation antigens.

Methods. Young adult (3- to 5-months old) hybrid recipients and parental donors of the following strains were used: (C57BL/Cum × 101/Cum)F_1, (C3H/Anf Cum × 101/Cum)F_1, (BALB/c Cum × A/He Cum) F_1, C57BL/Cum, C3H/Anf Cum, BALB/c Cum, A/He Cum, and 101/Cum; these are hereafter designated as $B1F_1$, $C31F_1$, CAF_1, C57, C3H, BALB/c, A, and 101, respectively. In every case, donor and recipient were of the same sex so that sex-determined histoincompatibility was avoided. Recipients were exposed to lethal whole-body irradiation as follows: strains $B1F_1$ and $C31F_1$ were given 900 r of X rays, and strain CAF_1, 800 r. The dose rate was approximately 85 r/minute; TSD, 93.5 cm; 300 kvp; 20 mA; 4.78 mm of Be inherent filtration and 3 mm of Al added filtration; hvl, 0.55 mm of Cu.

Hybrid mice were "immunized" against parental transplantation antigens by intraperitoneal inoculation of 20×10^6 nucleated viable parental spleen cells 30 and 15 days before irradiation. The parental spleen cells were obtained by teasing the spleen in Tyrode's solution with forceps and needles. The cell suspension was then passed through a stainless steel screen (200 mesh/inch) and the nucleated cells were counted in a hemocytometer.

Hybrid mice were also "immunized" by grafting of parental skin, the irradiation being carried out 100 days later without removal of the skin graft. Donor skin was taken from the ears and grafted onto the dorsal area of F_1 hybrid recipients by the method of Billingham and Medawar(13). The skin of the ear was used to avoid, insofar as possible, transplantation of dermal elements and included lymphoid cells. In every experiment, aliquots of a given parental marrow preparation were injected into each of the following groups of 10 recipients: (a) "nonimmunized" F_1 hybrids, (b) F_1 hybrids "immunized" against the parental donor strain, (c) F_1 hybrids "immunized" against the reciprocal parental strain. In addition, isologous marrow was given to F_1 hybrids "immunized" against one of the parental strains. The experiments were repeated to build up groups of 40 mice each, unless otherwise stated. Marrow cells were extruded from the femoral marrow cavity in Tyrode's solution by means of a syringe fitted with a 24-gauge needle. Recipients were inoculated with 10^7 nucleated cells by tail vein within 3 hours after irradiation. Characterization of hemoglobin type(14) in the $B1F_1$ recipients was carried out to ascertain whether donor type C57 red blood cells were present. Although irradiated marrow recipients were not serially sacrificed to follow pathological changes, dying mice in some of the experimental groups were sacrificed *in extremis* 20-90 days after treatment, and the following tissues were removed for histological examination: bone marrow from a long bone and the sternum, spleen, lymph nodes, thymus, Peyer's patches, intestine, liver, and kidney. Tissues of 16 B1F, 10 $C31F_1$, and 10 CAF_1 "immunized" mice were studied.

Results. In all donor-host combinations, the marrow protected against early lethality. Secondary mortality, however, occurred during the second and following months, varying in relation to strain and pretreatment of recipients (Fig. 1-3). In nearly all instances, secondary morbidity and mortality in F_1 recipients of parental marrow was accentuated by "immunization" against the donor strain; *i.e.,* by the preirradiation inoculation of parental lymphoid cells or grafting of parental skin. This effect was most marked in those strain combinations having parental H-2 histocompatibility differences proved to be significant for marrow transplantation(15), *i.e.,* the $B1F_1$ and the $C31F_1$ (Fig. 1 and 2). In the CAF_1 hybrid, preinoculation of A strain spleen cells increased secondary mortality only slightly (Fig. 3) but greatly increased the severity and frequency of secondary morbidity, *i.e.,* all of such recipients suffered marked weight loss, ruffling of fur, and epilation, changes which persisted until the end

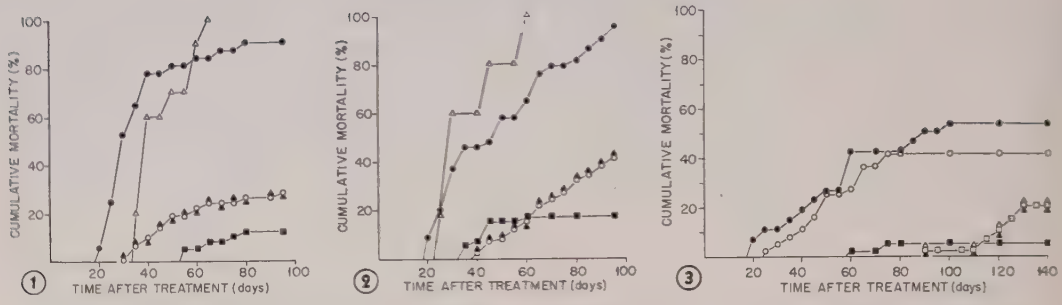

FIG. 1. Cumulative mortality of lethally irradiated $C31F_1$ mice after infusion of C3H or isologous marrow. Recipients were "immunized" against either parental spleen cells or skin. No. of animals in parentheses. ○ "Nonimmunized"; C3H marrow (40). ● C3H spleen; C3H marrow (40). △ C3H skin; C3H marrow (10). ▲ 101 spleen; C3H marrow (40). ■ C3H spleen; $C31F_1$ marrow (40).

FIG. 2. Cumulative mortality of lethally irradiated $B1F_1$ mice after infusion of C57 or isologous marrow. Recipients were "immunized" against either parental spleen cells or skin. No. of animals in parentheses. ○ "Nonimmunized"; C57 marrow (40). ● C57 spleen; C57 marrow (40). △ C57 skin; C57 marrow (10). ▲ 101 spleen; C57 marrow (40). ■ C57 spleen; $B1F_1$ marrow (40).

FIG. 3. Cumulative mortality of lethally irradiated CAF_1 mice after infusion of parental or isologous marrow. Recipients were "immunized" against either parental spleen cells or skin. No. of animals in parentheses. ○ "Nonimmunized"; A marrow (40). ● A spleen; A marrow (40). ■ A spleen; CAF_1 marrow (40). □ "Nonimmunized"; BALB/c marrow (40). ▲ BALB/c spleen; BALB/c marrow (40). △ BALB/c skin; BALB/c marrow (10).

of the observation period; in contrast, these symptoms were transient and mild in corresponding "nonimmunized" recipients of the same strain. In CAF_1 recipients of BALB/c marrow, secondary morbidity and mortality were minimal and unaffected by "immunization" against BALB/c cells (Fig. 3). In no combination did preirradiation inoculation of parental spleen cells homologous to the marrow donor strain affect the response of the recipients (Fig. 1-3). Hemoglobin in the peripheral blood of all 60-day and 80-day surviving $B1F_1$ recipients, whether "immunized" or not, was of donor type (70-100% of total Hb), indicating that the grafted erythropoietic cells were functional during the 80 days following parental marrow transplantation.

Histological preparations showed that the femoral and sternal marrow cavities were filled with nucleated cells, the marrow being at various stages of regeneration depending on the length of time elapsed from treatment. In no graft-host combination, even in "immunized" groups, was marrow necrosis noted. Lymph nodes and Peyer's patches of $B1F_1$ chimeras showed severe pathological changes similar to those described in the foreign bone marrow reaction(16), with atrophy and destruction of germinal centers, granulomatous proliferation of reticuloendothelial cells, and fibrosis; no difference was noted between "immunized" and "nonimmunized" recipients. The lymphoid tissues of the $C31F_1$ and CAF_1 chimeras showed mild changes, mainly atrophic in nature. They eventually recovered. Similarly, in these lymphoid tissues no differences were noted between "immunized" and "nonimmunized" recipients.

Discussion. The accentuation of secondary morbidity and mortality in irradiated F_1 hybrid recipients of parental marrow by pregrafting of donor-strain spleen cells or skin may be interpreted to mean that the grafting immunized against the donor marrow antigens. That this effect was immunological in nature is suggested by the correlation between its severity and the degree of histoincompatibility between the parental strains in question and by the lack of effect when nonspecific spleen cells (*i.e.*, cells other than those of the marrow donor strain) were preinoculated. Supporting data have been obtained in other experiments in which injection of isologous F_1 immunologically competent cells along with parental marrow into lethally-irradiated F_1 recipients leads to enhanced mortality, results suggesting that the F_1 hybrid cells reacted against parental mar-

row cell antigens(10,12). Also compatible with such an interpretation is the observation that isologous F_1 spleen (Cudkowicz, unpublished data) or marrow(17) cells inhibited the graft-versus-recipient reaction resulting from inoculation of parental liver cells into sublethally irradiated F_1 recipients (17).

The absence of enhanced morbidity and mortality in CAF_1 mice inoculated with BALB/c spleen cells and then given BALB/c marrow is tentatively ascribed to the lack of strong BALB/c histocompatibility antigens against which the CAF_1 hybrid can react; *i.e.*, the BALB/c is H-2^d in histocompatibility phenotype and possesses no major transplantation antigens foreign to the A strain (18,19).

Since the marrow was cellular and the circulating hemoglobin of donor type in moribund chimeras, death could not have resulted from rejection of the grafted parental marrow cells. The mechanism by which pregrafting accentuated secondary disease remains, therefore, to be elucidated. Death has been repeatedly observed, however, without necrosis of the marrow and spleen red pulp in parent-to-hybrid chimeras(9,20-24). In most of these cases the cellular and viable-appearing marrow was of host type, yet death was nevertheless ascribed to graft-versus-host immunological reactions; hence, other lesions resulting from *in vivo* antigen-antibody reactions are implicated and remain to be defined.

Enhanced secondary morbidity and mortality in the "immunized" F_1 chimeras might conceivably be attributed to aggravation of the graft-versus-host reaction by the inoculated parental lymphoid cells. This interpretation fails, however, to account for the similar effect of simultaneous injections of F_1 cells along with the parental marrow, and the lack of the effect in CAF_1 mice inoculated with BALB/c lymphoid cells. Furthermore, "immunization" of $B1F_1$ and $C31F_1$ hybrids could be accomplished just as effectively by skin grafting 100 days before irradiation as by inoculation of spleen cells. It is, therefore, inferred that the "immunizing action" did not require injection of viable donor-type antibody-forming cells and that it was not tissue-specific. "Immunization" has thus far failed to cause rejection of parental skin grafts in the strain combinations mentioned in this paper (Cudkowicz, unpublished data), suggesting that hemopoietic or lymphoid cells grafted into irradiated recipients may constitute a more sensitive indicator of histocompatibility differences than skin grafted onto unirradiated hosts.

There is now no direct evidence in the literature for active immunization of F_1 hybrid mice against parental transplantation antigens(7), the observed facts fulfilling generally the "one-autosomal gene, one antigen" theory. The occurrence of hybrid-anti-parent reactions, such as reported here, implies that parental antigen(s) are lacking in the hybrids between 2 inbred lines. Nevertheless, genic or allelic interactions in the inheritance of mouse histocompatibility factors have been postulated by many investigators on theoretical grounds or to explain certain experimental results(5-7,11,25) and have been demonstrated in species other than the mouse(7, a review). Their discovery in the mouse is, therefore, not altogether revolutionary.

Summary. 1. Previous grafting of parental spleen cells or skin accentuated secondary disease in F_1 hybrid mice subsequently irradiated and inoculated with marrow from donors of the same parental strain. 2. The intensity of the effect varied in relation to the extent of H-2 histocompatibility differences among the 4 strain combinations tested. 3. The effect is ascribed to "immunization" of the F_1 hybrid against parental tissue antigens. It is, therefore, inferred that parental histocompatibility alleles may not necessarily be expressed as codominants in F_1 hybrid mice.

Addendum. After submission of the manuscript, additional experiments showed that when the CAF_1 hybrid was "immunized" against A spleen cells by *three*, instead of two, inoculations at 30, 20, and 10 days prior to marrow grafting, respectively, secondary mortality *i.e.*, between 20-90 days) was enhanced to an extent comparable with that noted in the other strain combinations tested. However, the same treatment with BALB/c cells was not followed

by increased secondary mortality. These results indicate, therefore, that A strain parental tissues are also capable of "immunizing" F_1 hybrid mice.

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Effect of Stress on Potassium Content of Rat Brain. (26813)

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Previously it was shown that various types of injurious stimuli, when applied to peripheral tissues, cause a release of intracellular potassium(1), and that this release is prevented by analgesic agents(2). Adriani(3) suggests that process of excitation and inhibition in the C.N.S. may parallel those of peripheral mechanisms. A specific relationship between C.N.S. excitability and potassium (K) is indicated by the work of Ghosh and Quastel(4), Himwich(5), and Tsukeda (6). A number of workers found a low serum K during anesthesia(7,8,9). The present investigation represents an attempt to determine whether release and uptake of K by brain cells may be one aspect of the C.N.S. phase of the general stress response.

Methods. Rats were exposed to 3 different types of stress conditions: 1. (a) Anoxia was produced by evacuating a bell jar to an air pressure of 225 mm Hg. This was maintained for 120 minutes. (b) After return to ambient pressure, the animal was immediately decapitated. The brain was peeled out and washed in K-free Ringer solution. The 2 hemisections were separately macerated with fine sand, pestle and mortar. Potassium (K) content was determined from flame-photometric analysis of the watery extract and expressed as microequivalents per gram of dry tissue weight.

2. Heat stress was produced by putting a rat in a hot chamber at 42°C. The rat was maintained in the chamber until there were signs of impending collapse (air hunger). This took between 17 and 32 minutes with a

TABLE I. Effect of Stress on K Concentration of Rat Brain.

	Stimulus: 225 mm Hg	42°C	4°C	Control
	Time: 120 min.	23 min.	120 min.	
K conc.*	29.34	26.47	25.03	37.88
Significance†	.95	.99	.99	

* Microequivalents/g of dry tissue, mean of 8 tests.

† Difference experimental and control value, p value, (t test).

mean of 23 minutes. Subsequent procedures were the same as those under 1-(b).

3. Rats were kept in a refrigerator at 4°C for 120 minutes. The animals showed little effect of the cold (liveliness, eyes, fur). Subsequently, they were treated as those of the other groups 1-(b).

4. Controls were treated as under 1-(b).

Results are given in Table I. The differences between the individual stimuli are not significant. P values indicate degree of significance of the stimulus-control difference.

Conclusion. Systemic stresses of anoxia, heat and cold in rats result in a significant decrease of potassium concentration of the brain.

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Effect of Stress on Potassium Release from Surviving Rat Brain. (26814)

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In previous work(1) it was shown that various types of systemic stress (anoxia, heat, cold) decrease the potassium (K) concentration of the brain. This paper is an attempt to determine the importance of certain factors which may affect this potassium release.

In a first series of experiments guinea pigs were killed by a blow on the head. The brain was immediately taken out, hemisectioned and immersed in a bath of 10 ml of K-free mammalian Ringer's solution. While one half was exposed to cold (3°C) or heat (42°C) the other half was maintained at control level (23°C). Potassium release for the next 90 minutes was determined from the flame-photometric analysis of the bathing solution. Results are presented in Table I. It is assumed that the potassium loss as indicated by these data is limited to the most superficial layers of the hemisectioned brain.

Extreme heat has no significant effect on K release. Extreme cold produces a decrease of K release. As either stimulus, when applied systemically to the intact animal, results in a marked increase of K release from the brain(1), it appears that the systemic effect in the intact animal is not due to a direct temperature effect on the brain.

As another approach to this problem the following tests were performed on rats: The animal was decapitated, the brain immediately removed, hemisectioned and maintained in separate tubes containing K-free Ringer solution. Oxygen was bubbled through one test tube and nitrogen through the other.

TABLE I. Effect of Temperature on K Release of Surviving Brain Hemisections (Guinea Pigs).

Time, min.	4°C	23°C	42°C
0	3.62	4.23	4.43
30	8.34	9.75	10.05
60	11.63	13.54	13.98
90	13.19	15.58	16.06
△t	9.57	11.35	11.63

Each value is the mean of 8 tests. Units are microequivalent K released/g dry brain wt. t test analysis shows a significant difference ($p > .95$) 4°:23° and 4°:42°. △t gives K release during the 90 min. experimental run.

TABLE II. Effect of Anoxia on K Release of Surviving Brain Hemisections (Rats).

Time, min.	O_2	N_2
15	12.24	9.50
35	20.59	22.83
55	23.71	32.25
△t	11.47	22.75

Values are microequivalents K released/g dry wt. t test analysis shows significant difference ($p > .99$).
Each value is the mean of 20 tests.
△t gives the K release during the 40 min. experimental run.

Subsequent procedures were the same as in previous experiments. The mechanical agitation by the gas bubbles increases the release of K. However, the difference between the two conditions (Table II) indicates that anoxia has a direct effect on K release of brain tissue.

In the next experiment, hemisections of the brains of 10 rats were exposed for 15 minutes to glucose-free Ringer's solution, while the other halves served as controls. Mean control value was 3.84 microequiv. per gram and that of the glucose free solution was 19.90. The difference is highly significant ($p > 0.99$). This is considered an indication that glucose is needed for maintenance of intracellular potassium.

Table III gives the effect of 4 selected drugs on K release. While complete data for 90 minutes analysis are available, listed values are only those obtained after one hour. The results indicate that epinephrine may play a role in the increased K release of stress. Surprising to us was the great increase obtained with gamma-amino-butyric-acid, and the negative results obtained with acetylcholine, while the inhibition observed with nembutal could be expected.

Conclusion. Using *in vitro* preparations of rat brains it was found that temperature had little direct effect on K release. Oxygen and glucose are necessary to maintain K within the brain cells. Acetylcholine has no effect on K release, epinephrine and gamma-amino-butyric-acid increase the release, while nembutal acts as an inhibitor.

TABLE III. Effect of Drugs on *In Vitro* K Release of Brain (Rats) within 60 Minutes.

Acetylcholine		Epinephrine		Nembutal		Gamma-amino butyric acid	
Drug conc.*	K conc.†	Drug conc.*	K conc.†	Drug conc.*	K conc.†	Drug conc.*	K conc.†
10	12.49	10	17.93	6.0	9.63	50	24.90
2.0	12.60	2.0	15.79	1.2	11.01	10	19.84
.4	12.46	.4	14.33	.24	12.63	2	16.01
0	12.89	0	12.89	0	12.89	.4	13.70
						0	12.89

* Drug concentrations are mg/ml.
† K concentrations are microequivalents released/g dry tissue.
Each value is the mean of 8 tests. The increase with epinephrine and GABA, as well as the decrease with nembutal are significant ($p > .95$—t test).

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Influence of Nicotinic Acid and Nicotinamide on Insulin Hypoglycemia.* (26815)

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Blood sugar concentration can be altered by administration of either nicotinic acid or nicotinamide, but the direction of the response induced apparently depends primarily on the species, agent and dosage used. Both nicotinic acid and nicotinamide have been found to increase blood sugar concentration in the rat and rabbit(1,2). Mirsky *et al.*, however, obtained a marked hypoglycemia with nicotinic acid in the rat and the mouse(3). Hypoglycemia has also been produced in man with nicotinamide(4), although most investigators have not found it to cause any significant change in blood sugar concentration(5,6). The present study delineates the action of various doses of these compounds in normal and insulin-treated mice.

Materials and methods. Male Swiss-Webster mice weighing from 18 to 24 g were maintained in an air-conditioned room at 26°C. Crystalline zinc insulin (40 units/ml) was diluted with physiological saline and sufficient hydrochloric acid added to adjust the final pH to 2.5-3.0. It was administered intraperitoneally in a dose of 5 units/kg. Nicotinic acid or nicotinamide (500 mg/kg) was administered subcutaneously. The nicotinic acid was adjusted to pH 7 with $NaHCO_3$. All solutions were administered in a volume of 0.1 ml per 10 grams of body weight. Physiological saline was used as control.

Animals previously maintained on normal mouse diet with water *ad libitum* were fasted 22 to 24 hours and then divided by random selection into the various groups. Onset of hypoglycemic convulsions was determined by a modification of the sloped screen technic of Thompson(7). Blood sugar was initially determined by the method of Nelson(8) and later by an enzymatic method(9) without any significant change in the values obtained. At approximately half-hour intervals, 0.2 ml of blood was drawn from the orbital sinus of individual mice in each group(10). No animal had blood taken more than once.

TABLE I. Hypoglycemic Falls in Insulin-Treated Mice.

Drugs	No. of mice	% falls within 150 min.
Insulin + .9% NaCl	50	54%
" + nicotinic acid	50	90
" + nicotinamide	50	14
" + nicotinamide and nicotinic acid	20	15
Nicotinamide + .9% NaCl	15	0
Nicotinic acid + " "	15	0

Insulin (5 units/kg, I.P.); nicotinic acid and nicotinamide (500 mg/kg, subcut.).

P (x^2) Insulin *vs* insulin + nicotinic acid = <.001.

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P (x^2) Insulin + nicotinic acid *vs* insulin + nicotinamide = <.001.

Results. The percentage of animals in each group falling from the inclined screen within 150 minutes after injection is given in Table I. A cut-off time of 150 minutes was chosen because approximately 75% of exogenous insulin is known to be metabolized in mice by that time(11). Neither nicotinic acid nor nicotinamide alone caused any mice to fall. Of the animals showing hypoglycemic falls with insulin alone, the majority fell within the first 90 minutes. Administration of both nicotinic acid and insulin resulted in a significant increase in the number of animals falling. Nicotinamide on the other hand caused a significant decrease in the number of insulin-treated animals falling. Earlier falls were noted (the majority within 60 minutes) in mice treated with insulin plus either nicotinic acid or nicotinamide.

Saline-injected controls had an average blood sugar level of 70.9% (Table II). Insulin lowered blood sugar by 78% within from 1.5 to 2 hours. Nicotinamide (500 mg/kg) elevated the level to a maximum of 32% over control values at 2 to 2.5 hours after

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TABLE II. Blood Sugar Concentration* in Insulin-Treated Mice.

Group	Drug	Dose, mg/kg	Minutes after administration					
			0-30	30-60	60-90	90-120	120-150	150-180
1	.9% NaCl	—	70.4 ± 3.3 (10)	67.1 ± 2.4 (10)	71.8 ± 2.3 (10)	74.5 ± 2.8 (10)	72.6 ± 2.5 (10)	69.1 ± 2.7 (10)
2	Nicotinic acid + .9% NaCl	500	—	65.2 ± 3.4 (5)	60.0 ± 3.7 (5)	56.1 ± 3.1 (5)	59.2 ± 3.5 (5)	66.2 ± 3.3 (5)
3	Nicotinamide + .9% NaCl	500	—	76.4 ± 3.8 (5)	83.1 ± 4.1 (5)	89.3 ± 3.6 (5)	96.1 ± 3.8 (5)	85.3 ± 3.2 (5)
4	Insulin + .9% NaCl	—	32.2 ± 2.9 (10)	27.6 ± 2.2 (10)	22.3 ± 2.5 (10)	16.2 ± 3.2 (10)	29.7 ± 2.3 (10)	— —
5	" + nicotinic acid	500	24.3 ± 2.4 (10)	19.1 ± 2.3 (10)	14.0 ± 2.5 (10)	9.4 ± 2.5 (10)	21.1 ± 2.8 (10)	— —
6	" + nicotinamide	500	38.1 ± 2.6 (10)	40.1 ± 2.8 (10)	36.3 ± 2.3 (10)	38.8 ± 3.3 (10)	43.7 ± 3.2 (10)	— —
7	*Idem*	250	28.2 ± 3.1 (5)	30.2 ± 3.2 (5)	24.9 ± 3.4 (5)	21.9 ± 3.3 (5)	28.5 ± 3.5 (5)	— —
8	"	125	25.2 ± 3.4 (5)	27.7 ± 3.1 (5)	15.8 ± 3.6 (5)	12.2 ± 3.6 (5)	24.2 ± 3.1 (5)	— —

* Blood sugar concentration in mg % ± stand. dev. (No. of mice/group).
Insulin = 5 units/kg, i.p.; other agents, subcut.

administration. This dosage also caused a significant elevation in blood sugar concentration in insulin-treated mice. When the dose of nicotinamide was decreased to 250 mg/kg, the level was not significantly different from that of the insulin-treated group. With a further decrease in dosage to 125 mg/kg the blood sugar concentration tended to be lower than that of the insulin-treated controls. By contrast, nicotinic acid (500 mg/kg) produced a significant decrease in blood sugar concentration in both control and insulin-treated mice.

Discussion. It is known that administration of 500 mg/kg of nicotinamide can significantly increase the level of active DPN in mice(12). However, since maximum changes in DPN levels do not occur until 8-12 hours after administration of the vitamin, it does not seem likely that the rapid effect demonstrated in the present study depends on the same action. Mirsky *et al.* have found that nicotinic acid at this dosage inhibits insulinase activity while nicotinamide increases insulinase activity(13). Since nicotinamide produced the same increase in blood sugar level over both control and insulin-treated animals, the nicotinamide effect was apparently independent of the level of circulating insulin. This suggests that the action was on other than on an insulin dependent system. Although the exact mechanism of action by which the vitamin influences blood sugar concentration is unknown, the present study demonstrates that in mice, high doses of nicotinic acid and nicotinamide have opposite effects on blood sugar whether given alone or in combination with insulin. At a lower dose nicotinamide mimics nicotinic acid in its effect on blood sugar concentration.

Summary. Nicotinamide (500 mg/kg) elevates blood sugar in both normal and insulin-treated mice and decreases the incidence of hypoglycemic convulsions in the latter. At 125 mg/kg, however, nicotinamide increases the depth of insulin hypoglycemia. Nicotinic acid (500 mg/kg) lowers blood sugar concentration in both control and insulin-treated mice and increases the incidence of hypoglycemic convulsions.

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Effect of Dietary Vitamin B_{12} on Methionine Biosynthesis by Chick Liver Homogenates.* (26816)

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It was observed several years ago that dietary vitamin B_{12} would spare the methyl requirements for animals(1). More recently, considerable evidence has accumulated to implicate Vit. B_{12} in methionine biosynthesis in bacterial systems(2,3). Arnstein(4) has demonstrated in *in vivo* experiments with rats that Vit. B_{12} stimulates the conversion of formate to the methyl groups of choline and methionine. We have reported(5,6) that Vit. B_{12} stimulates the conversion of formate to the 5-methyl group of thymine by chick bone marrow cells but does not stimulate conversion of formaldehyde to the 5-methyl group of thymine. These results suggest that Vit. B_{12} functions in reduction of one-carbon compounds between the formate and formaldehyde levels of oxidation. In the present experiments we have studied the effect of Vit. B_{12} deficiency in chicks on formation of the methyl group of methionine by liver homogenates and the results suggest that B_{12} stimulates the conversion of formate to methionine methyl but does not increase conversion of formaldehyde to methionine methyl, a situation analogous to our previously reported results in thymine formation by chick bone marrow cells.

Experimental. Day-old White Leghorn chicks were given the Vit. B_{12} and methionine-deficient basal diet described by Spivey-Fox *et al.*(7). When present, methionine was added at a concentration of 6% of the diet and Vit. B_{12} was given by weekly injections at a dosage of 3 μg per chick the first week and 9 μg per chick thereafter. After 3 to 4 weeks on the various diets, the chicks were taken for experiment. Peripheral blood counts were made using standard hematological procedures.

Methionine formation from homocysteine and formate-C^{14} or formaldehyde-C^{14} was determined in liver homogenates. The livers were homogenized in an all-glass homogenizer with 2 volumes of Robinson's buffer(8). The incubation mixture consisted of 2 ml of homogenate, 3 mg of DL-homocysteine, and either 0.27 μmole of formate-C^{14} (142,000 c.p.m.) or 2 μmoles of formaldehyde-C^{14} (140,000 c.p.m.). The final volume was 2.5 ml. After an hour of incubation at 37° under air in the Dubnoff shaker, the reaction was stopped by addition of 0.5 ml of 50% trichloroacetic acid and the contents of the incubation beakers were transferred to 12 ml conical centrifuge tubes and centrifuged. 1.5

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TABLE I. Effects of Dietary Supplements of Methionine and Vitamin B_{12} on Growth and Hemogram of Chicks.

Supplement	Weight gain, g/4 wk	Erythrocytes, millions/μl	Hemoglobin, g/100 ml	Hematocrit, %
None	154	2.83	9.6	29
Methionine	241	2.70	9.1	29
B_{12}	237	2.83	9.5	29
Methionine + B_{12}	250	3.04	9.7	31

ml of the supernatant solution was taken and 200 mg of carrier L-methionine added and dissolved with heating. The methionine was precipitated on cooling with addition of 95% ethanol and recrystallized in this fashion a total of 6 times. The methionine was then plated on aluminum planchets and counted at infinite thickness with an end-window Geiger counter. The results are reported as counts per minute of the infinite thickness samples. To correct for any non-enzymatic reaction between formaldehyde, formate and homocysteine zero time samples were taken and the counts obtained subtracted from the counts obtained in the incubated samples. Five chicks from each group were used in these experiments.

Results and discussion. Supplements of either B_{12} or methionine stimulated growth of chicks as indicated by the data in Table I. In the presence of adequate methionine B_{12} produced no significant effect on the growth rate. These data again point out the well-known effect of Vit. B_{12} in sparing the methyl requirements of animals. The hemogram of the chicks was not significantly influenced by B_{12} deprivation indicating the mildness of the B_{12} deficiency.

TABLE II. Influence of Dietary Vitamin B_{12} and Methionine on Formation of Methionine Methyl by Chick Liver Homogenates. Results reflect total C^{14} incorporated into methionine. See text for details of calculation.

Supplement	C^{14} substrate employed: Formate	Formaldehyde
None	239*	43†
B_{12}	511	12
Methionine	237	44
Methionine + B_{12}	353	25

* B_{12} effect statistically significant. $P < .01$. Methionine effect not statistically significant.

† B_{12} effect statistically significant. $P < .05$. Methionine effect not statistically significant.

Statistically treated by analysis of variance.

Vit. B_{12} stimulated the conversion of formate to methionine methyl but appeared to inhibit conversion of formaldehyde to methionine methyl as indicated by the data in Table II. This observation is in agreement with studies of the influence of Vit. B_{12} on biosynthesis of the methyl group of thymine (5,6). Methionine supplementation did not exert a statistically significant effect on methionine biosynthesis by liver homogenates.

These results again demonstrate the involvement of Vit. B_{12} in methyl synthesis by animals and substantiate our earlier suggestions concerning the site of action of B_{12}. The results indicate that Vit. B_{12} functions between the formate and formaldehyde levels of oxidation, a reaction catalyzed by the enzyme methylene tetrahydrofolic dehydrogenase. We have previously reported a reduction in activity of this enzyme in tissues from B_{12} deficient animals(9).

Summary. Liver homogenates from B_{12}-deficient chicks converted formate to methionine methyl at a reduced rate and converted formaldehyde to methionine methyl at an accelerated rate. These results suggest the involvement of Vit. B_{12} in reduction of formate to formaldehyde, a reaction mediated by methylene tetrahydrofolic dehydrogenase.

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Nutritional Equivalence of β-Lactoglobulin and Its Corresponding Amino Acids. (26817)

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One of the most important problems in protein metabolism is the comparison of the nutritional behavior of a protein with that of its constituent amino acids. Many growth studies utilizing amino acids have been reported, but to the authors' knowledge in no case has a comparison been made with a crystalline protein, free from carbohydrate, phosphorus, prosthetic groups and possible unidentified contaminants which might possess growth stimulating properties. A frequent comparison has been with casein, which obviously does not fill the above requisites and in such studies the amino acid mixture does not provide growth equal to that obtained with the intact protein(1,2,3). During the period 1945 to 1948 studies with crystalline β-lactoglobulin were conducted with mice. The difficulties in obtaining sufficient quantities of the crystalline protein necessitated small groups of animals and these early studies were not reported except for an ACS meeting abstract (Sept. 1948). It has been hoped that an extension of this work would provide valuable additional data, but the importance of the original observations is so great that results of these experiments are being presented even though expanded observations have not been possible.

Materials and methods. Crystalline β-lactoglobulin was made from fresh raw skim milk by modification of the procedure of Palmer(4) adapted for large scale isolation. The purity of the β-lactoglobulin was established by electrophoretic homogeneity at pH 4.8 and 6.5(5). The crystalline protein was maintained wet and under toluene until all measurements of purity were complete and was then dried by lyophilization before incorporating into diets. The amino acid composition described by Brand *et al.*(6) was used in preparing the amino acid counterpart shown in Table I. Amino acids were purchased from commercial suppliers and purity was established by checking optical rotation and melting points. Since basic amino acids were to be fed as their hydrochlorides, a calculated equivalence of $NaHCO_3$ was added to the mixture (Table I).

Diet composition is given in Table II. In addition to β-lactoglobulin and its amino acid counterpart, 2 crude protein sources, a vitamin test casein and a commercial lactalbumin, were used as additional controls. All proteins were incorporated in the diets at equal nitrogen levels and amounts used were selected to give growth rates that would be somewhat less than the maximum achievable by the mouse. As closely as possible, by calculation, the diets were isocaloric. Sodium chloride was added to the protein-containing diets to balance the sodium of the $NaHCO_3$ in the amino acid diet.

Since mice, as well as other animals, must adjust to diets containing amino acids, the amino acid diet was fed for 3 days to 30 male weanling mice selected for uniformity of weight at 18 days of age (Swiss-Webster Strain, Sharp and Dohme Colony). During the adjustment period 1.5% of 1:20 liver powder was incorporated in the diet. These experiments were conducted prior to crystallization of Vit. B_{12}, but it was recognized that a source of an unidentified factor was required by the mouse. In the light of subsequent studies it is probable that during the preweanling period and with the liver powder fed during the 3 day adjustment period, sufficient stores of Vit. B_{12} were accumulated so that the absence of this vitamin during the subsequent 4 week test was not limiting for the animals. Sixteen mice that showed uniform growth responses during the 3 day adjustment period were divided into 4 groups of 4 mice each with equalized weight distribution. The mice were caged individually and

* Deceased.

TABLE I. Amino Acid Composition of β-lactoglobulin and Amino Acid Mixture.

	β-lactoglobulin		Amino acid mixture		
	Amino acid yield/100 g protein, g	N/100 g protein, g		Amino acid, g	N, g
Glycine	1.4	.26		1.4	.26
Alanine	(7.8)*	(1.23)*	L	7.8	1.23
Leucine	15.6	1.67	L	15.6	1.67
Proline	4.1	.50	L	4.1	.50
Cysteine	1.11	.13	—	—	—
Cystine	2.29	.27	L	3.4	.40
Tryptophan	1.94	.27	L	2.0	.28
Tyrosine	3.8	.29	L	3.8	.29
	38.04	4.62		38.10	4.63
Arginine	2.88	.93	L-HCl	3.5	.93
Histidine	1.58	.43	L-HCl-H_2O	2.1	.43
Lysine	11.40	2.18	L-HCl-H_2O	15.6	2.18
	15.86	3.54		21.2	3.54
Aspartic acid	11.4	1.20	L	3.3	.35
Asparagine	—	—	L	7.9	1.67
Glutamic acid	19.5	1.86	L	17.1	1.63
Glutamine	—	—	L	2.2	.42
Methionine	3.22	.30	L	3.2	.30
Phenylalanine	3.54	.30	L	3.5	.30
Valine	5.8	.70	L	5.8	.70
Isoleucine	(6.0)*	(.64)*	L	6.0	.64
Serine	5.0	.67	L	5.0	.67
Threonine	5.85	.69	L	5.85	.69
	60.31	6.36		59.85	7.37
Amide NH_3	1.3	1.08			
Na HCO_3				8.7	
Total	115.51	15.60		127.85	15.54
	15.60/115.51 = 13.50%			15.54/127.85 = 12.16%	

* Values revised from those given in original references.

TABLE II. Composition of Diets.

	Holding	A	B	C	D
	g				
Casein[1]	—	17.0	—	—	—
Lactalbumin[2]	—	—	19.5	—	—
β-lactoglobulin	—	—	—	15.3	—
Amino acid mixture	19.0	—	—	—	19.2
Hydrogenated cottonseed oil[3]	25	25	25	25	25
Corn oil[4]	2	2	2	2	2
Glucose[5]	20	20	20	20	20
Salt mixture[6]	4	4	4	4	4
Cellulose[7]	2	2	2	2	2
1:20 liver conc. powder	1.5	—	—	—	—
Choline chloride	.2	.2	.2	.2	.2
Sodium chloride	—	.9	.9	.9	—
White dextrin	26.3	28.9	26.4	30.6	27.6
Total	100.0	100.0	100.0	100.0	100.0
% nitrogen in diet	2.33	2.34	2.34	2.34	2.53

Each diet supplemented to contain per 100 g: 4 mg of α-tocopherol, 900 U.S.P. units of vit. A, 180 U.S.P. units of vit. D, 1 mg of 2-methyl-1,4-naphthoquinone diacetate, 0.8 mg of thiamine HCl, 1.6 mg of riboflavin, 0.8 mg of pyridoxine HCl, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, and 21.6 mg of inositol.

fed *ad libitum* in a temperature controlled room. Daily weights and food consumption were measured.

Results and discussion. Performance data at 14 and 28 days are given in Table III. Growth rate and efficiency of nitrogen utilization were the same for the crystalline β-lactoglobulin and its corresponding amino acid mixture. On the other hand, these 2 groups appear to have had lower growth rates and ratios of gain to nitrogen intake than was obtained with the 2 crude proteins, casein

Footnotes: [1] Bordens Labco, [2] Bordens 15-42, [3] Primex, [4] Mazola, [5] Cerelose, [6] Jones, J. H., and Foster, C., *J. Nut.*, 1942, v24, 245; [7] Cellu Flour.

TABLE III. Growth and Nitrogen Utilization of Mice Fed Diets Containing Intact Proteins and Amino Acid Mixture.

	Initial wt, g	14 days			28 days		
		Wt gain, g	N intake, g	Wt gain / N intake	Wt gain, g	N intake, g	Wt gain / N intake
Casein	9.9	11.3	1.00	11.3	12.6	2.03	6.2
	9.7	10.8	1.08	10.0	12.8	2.15	6.0
	9.5	9.4	1.00	9.4	11.8	2.03	5.8
	8.9	12.1	.98	12.3	15.5	2.15	7.2
Avg	9.5	10.9	1.02	10.7	13.2	2.10	6.3
Lactalbumin	12.1	7.1	.91	7.8	9.3	1.96	4.7
	8.5	13.3	1.08	12.3	16.2	2.32	7.0
	8.6	11.9	1.03	11.6	13.1	2.11	6.2
	9.1	9.4	.90	10.4	13.3	1.94	6.9
Avg	9.6	10.4	.98	10.5	13.0	2.08	6.2
β-lactoglobulin	8.2	7.9	.75	10.5	10.7	1.79	6.0
	9.9	10.6	.99	10.7	12.0	2.10	5.7
	9.4	9.0	1.00	9.0	11.4	2.08	5.5
	10.6	8.6	.99	8.7	11.0	1.94	5.7
Avg	9.5	9.1	.93	9.7	11.3	1.98	5.7
Amino acid mix	9.4	7.0	.81	8.6	11.3	1.84	6.1
	9.4	8.5	.97	8.8	12.6	2.15	5.9
	9.1	9.7	1.02	9.5	11.8	2.31	5.1
	9.9	8.8	.99	8.9	11.2	2.08	5.4
Avg	9.4	8.5	.95	9.0	11.7	2.09	5.6

and lactalbumin. Since small groups had to be used, calculations of significance of differences does not seem warranted and therefore individual results have been given. It will be very important to ascertain whether the apparent difference between β-lactoglobulin and the crude proteins is a reflection of amino acid pattern or some other factor.

In numerous comparisons of growth responses to crude proteins and amino acid mixtures, the proteins have been superior. In the present studies a crystalline protein, free from carbohydrate, phosphorus, prosthetic groups and unidentified constituents gave equivalent growth response to an amino acid mixture corresponding exactly to the native protein. This is probably the only time a comparison has been made in which the complete amino acid composition of a protein has been accurately reproduced. In spite of the extremely high cost of this type of investigation, this model experiment must be extended to establish a complete understanding of the nutritional contributions of proteins and amino acids.

Summary. In mouse growth studies crystalline β-lactoglobulin has been compared with a mixture of L amino acids duplicating its composition and these materials were compared with casein and lactalbumin. The pure protein and its amino acid counterpart gave equal growth and efficiency of dietary nitrogen utilization. Casein and lactalbumin provided superior growth rates to both the pure protein and its amino acid counterpart.

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Studies on DNAase-Sensitive Antigens of *Brucella abortus* by Complement-Fixation.* (26818)

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Phillips, Braun, and Plescia reported that the sera of rabbits injected intravenously with a DNA-rich preparation from *Brucella abortus*, extracted by 0.5% phenol in citrate-saline, contained antibodies against DNAase-sensitive antigens(1). In addition, these sera, tested either by the Ouchterlony gel-diffusion technic(2) or the quantitative precipitin method of Heidelberger and Kendall(3), contained antibodies against other antigens(4). In a continuing study, over 100 rabbits were immunized with DNA-rich preparations obtained by the procedure described by Braun *et al.*(5). All of the immune sera contained precipitins and it was assumed, as before, that some of these precipitins were directed against DNAase-sensitive antigens. However, on further examination of these sera it was found that less than 5% of them had the desired antibodies. The irregularity with which antisera containing antibodies against DNAase-sensitive antigens were produced and its dwindling supply necessitated the use of a method requiring far less serum than the quantitative precipitin method. Consequently, the use of complement-fixation was explored and the results are summarized in this paper.

Materials and methods. Sheep blood was obtained at a local slaughter house, and sensitized sheep cells were prepared according to the procedure described by Plescia *et al.*(6). Unless otherwise indicated, veronal-saline buffer with optimal concentrations of Ca^{++} and Mg^{++}(7) was used for all washings and dilutions. Whole guinea pig serum was used as a source of complement. Individual sera were pooled, divided into small portions and stored at −45°C.

Rabbits were immunized at regular intervals over a 4-week period with different amounts of DNA-rich preparations(5), employing different routes. Those receiving intravenously 100 mg in terms of DNA gave best results.

Complement-fixation tests were done in duplicate with the reactants added in the following order: 0.2 ml of antiserum or buffer, 0.1 ml of guinea pig complement diluted 1/40 and 0.2 ml of antigen or buffer. The reaction mixture was incubated for 1 hour at room temperature and kept overnight at 4°C before adding 1×10^8 sensitized cells to each tube and placing it in a waterbath at 37°C for 1 hr with mixing to keep the cells suspended. Then 2.0 ml of buffer were added to each tube, and the mixtures were centrifuged in a Clay-Adams clinical centrifuge at 2,000 r.p.m. for 6 minutes. Optical densities of the supernatants were determined at 541 mμ, using a model B Beckman spectrophotometer.

Precipitation analysis in agar gel was done according to the procedure of Ouchterlony (2). Noble Agar (Difco) was used at a concentration of 0.85% in physiological saline. Merthiolate (0.01%) was added as a preservative. The agar gel plates were prepared and stored at 4°C about a week before using. The wells were then filled with the appropriate reactants, and plates kept at 26°C for a week to 10 days before final readings were made.

Worthington 1x crystallized pancreatic DNAase was used for enzymatic digestion of DNA with a ratio of substrate to enzyme of 10:1 and $MgSO_4$ to give a final concentration of 0.01 *M*. Reactions were carried out at 37°C for 24 hrs.

Results. Titration of guinea pig complement. The complement-fixation test was carried out with the minimum amount of guinea pig serum giving complete lysis of sensitized sheep erythrocytes. To determine this amount, different amounts of guinea pig serum were used giving extents of hemolysis ranging from 0-100%. Such a titration (Fig.

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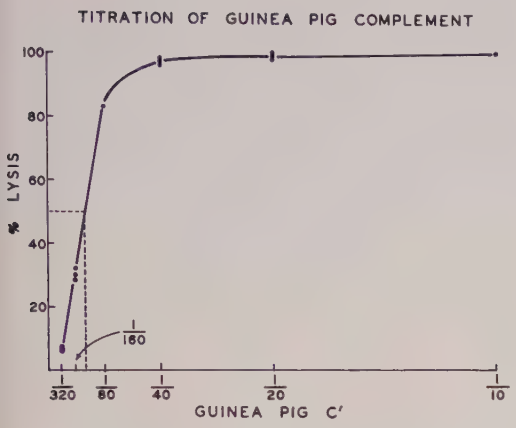

FIG. 1. Titration of guinea pig complement. Each determination was carried out in triplicate.

1) also indicated the sensitivity of the method and degree of reproducibility. On the basis of this titration, a dilution of 1:40 of guinea pig serum was used routinely. Results were reproducible throughout the entire range with a mean deviation of ± 2%. Sensitivity was uniform and greatest from 20-80%.

Analysis of antiserum for antibodies against DNAase-sensitive antigens. As an example of the antigen-antibody reactions studied, data obtained with antiserum #51 and DNA preparation #20 may be cited. The results of a gel-diffusion analysis are shown in Fig. 2. Whereas the DNAase itself showed no reaction with the antiserum, the control preparations, preincubated for 24 hours at 4°C or 37°C, showed several apparently similar bands of precipitation. Of these, the band closest to the antigen well was absent in the reaction between the antiserum and the preparation treated with DNAase at 37°C for 24 hours, indicating that some of the antibodies were directed against DNAase sensitive antigens. The reaction between antiserum #51 and DNA preparation #20 was also analyzed by complement-fixation (Table I). The antiserum was used at dilutions of

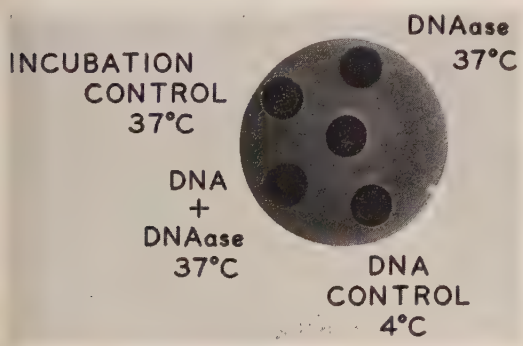

FIG. 2. Gel-diffusion analysis of rabbit anti-Brucella DNA serum #51 for antibodies against DNAase-sensitive antigens. Undiluted antiserum was placed in center well, different antigen preparations in outer well. Concentration of original DNA was .7 mg/ml.

TABLE I. Effect of DNAase on Antigens of *Brucella abortus* Tested by Complement-Fixation.

#20 preparation, 4°C

Antigen,* μg DNA	Antiserum #51 1:1000	1:100	Buffer
	%		
.02	102†	99	100
.2	100	80	100
2.0	36	1	98
Buffer	100	100	100

* U.V. absorption at 260 mμ and based on purified calf thymus DNA as standard.

† Represents % cells lysed. Extent of complement fixation is inversely proportional to % of cells lysed.

#20 preparation, 37°C, 24 hr

Antigen, μg DNA	Antiserum #51 1:1000	1:100	Buffer
	%		
.02	101	102	100
.2	100	74	101
2.0	51	0	99
Buffer	100	100	100

#20 preparation, DNAase, 37°C, 24 hr

Antigen, μg DNA	Antiserum #51 1:1000	1:100	Buffer
	%		
.02	99	100	102
.2	102	98	101
2.0	98	1	103
Buffer	101	100	100

1:100 and 1:1000. At the higher concentration of antiserum considerably more fixation of complement occurred than at 1:1000, and DNAase treatment of the antigen appeared to have no effect. With antiserum diluted 1:1000 there was somewhat less fixation of complement but the effects of DNAase now became apparent because antibodies to antigens other than the DNAase-sensitive ones

TABLE II. Effect of DNAase on DNA Preparation #34 of *Brucella abortus* Tested by Complement-Fixation with Standard Antiserum #51.

#34 preparation, 4°C		
Antigen,* μg DNA	Antiserum #51 1:500	Buffer
	%	%
.12	100†	100
.6	99	100
1.1	71	103
2.2	33	99
Buffer	99	100

* Absorption at 260 mμ and based on purified calf thymus DNA as standard.
† % cells lysed.

#34 preparation, DNAase, 37°C, 24 hr		
Antigen, μg DNA	Antiserum #51 1:500	Buffer
	%	%
.12	100	100
.6	97	99
1.1	85	102
2.2	42	100
Buffer	100	100

had apparently been diluted out. Thus antibodies to DNAase-sensitive antigens in serum #51 were demonstrated by both precipitation in agar gel and complement-fixation.

Analysis of DNA preparations for DNAase-sensitive antigens by complement-fixation. Antiserum #51, having been shown to contain antibodies against DNAase-sensitive antigens, was used for detection of DNAase-sensitive antigens in different lots of Brucella DNA preparations. Table II shows the results with DNA preparation #34. Antiserum was diluted 1:500 instead of 1:1000 because there was essentially no fixation at 1:1000, presumably because this preparation did not contain DNAase-sensitive antigens. This was indeed the case, because at the dilution of 1:500 complement was fixed but pretreatment of the DNA preparation with DNAase had no effect. Similarly, no DNAase-sensitive antigens could be detected in this preparation by means of precipitation in agar gel.

Table III gives the results of a similar analysis employing DNA preparation 1C and antiserum #51. As in the analysis of preparation #34, the antiserum was diluted 1:500. Considerable fixation of complement occurred, and treatment of the antigen preparation with DNAase had a significant effect indicating the presence of DNAase-sensitive antigens.

Analysis of antisera for antibodies against DNAase-sensitive antigens by complement-fixation. DNA preparation 1C, shown to contain DNAase-sensitive antigens, was next used as a standard for detection of antibodies against DNAase-sensitive antigens. Two antisera, #88 and S-12, were examined. The results are shown in Tables IV and V. With antiserum #88, pre-treatment of the DNA with DNAase had little, if any, effect, in contrast to the results with antiserum S-12.

Discussion. The foregoing results clearly indicate that not all the antibodies in a given antiserum against a DNA-rich preparation are directed against DNAase-sensitive antigens. Such antibodies, when present, have been shown to be detectable by complement-fixation provided their concentrations are sufficiently great to permit the diluting out of antibodies directed against other antigens. In sera in which the fraction of antibodies against DNAase-sensitive antigens is small,

TABLE III. Effect of DNAase on DNA Preparation #1C of *Brucella abortus* Tested by Complement-Fixation.

#1C preparation, 4°C		
Antigen,* μg DNA	Antiserum #51 1:500	Buffer
	%	%
.05	99†	100
.1	95	101
.5	81	101
1.0	32	102
2.0	18	102
Buffer	100	100

* U.V. absorption at 260 mμ and based on purified calf thymus DNA as standard.
† % cell lysis.

#1C preparation, DNAase, 37°C, 24 hr		
Antigen, μg DNA	Antiserum #51 1:500	Buffer
	%	%
.05	101	101
.1	98	102
.5	97	103
1.0	88	102
2.0	60	102
Buffer	100	100

complement-fixation may still be used; however, it would be necessary first to absorb the antiserum with antigens other than those sensitive to DNAase. Such tests are now in progress.

The foregoing results also demonstrate the existence of considerable variation among different DNA preparations and among different antisera produced against such preparations. Having obtained antiserum #51, shown to contain antibodies against DNAase-sensitive antigens, it became possible to pretest DNA preparations for presence of DNAase-sensitive antigens. This resulted in the selection of an immunizing preparation containing an appreciable amount of DNAase-sensitive antigens, and when rabbits were injected with this material, over 50% of them produced antibodies against DNAase-sensitive antigens. Previously, when immunizing preparations were not selected for DNAase-sensitive antigens, less than 5% of the animals produced sera that contained the desired antibodies. However, additional controlled experiments will be needed before it can be concluded that production of antibodies to DNAase-sensitive antigens depends upon the detectable presence of such antigens in the immunizing preparation.

TABLE IV. Testing of Antiserum for Antibodies against DNAase-Sensitive Antigens by Complement-Fixation with Preparation #1C Known to Contain DNAase-Sensitive Antigens.

#1C preparation, 4°C		
Antigen,* μg DNA	Antiserum #88 1:500	Buffer
	%	%
.05	99†	102
.1	97	100
.5	88	101
1.0	76	101
2.0	65	101
4.0	36	102
8.0	47	102
Buffer	99	100

* U.V. absorption at 260 mμ and based on purified calf thymus DNA as standard.
† % lysis.

#1C preparation, DNAase, 37°C, 24 hr		
Antigen, μg DNA	Antiserum #88 1:500	Buffer
	%	%
.05	99	102
.1	100	100
.5	86	98
1.0	75	102
2.0	54	102
4.0	42	102
8.0	54	102
Buffer	100	100

TABLE V. Testing of Antiserum for Antibodies against DNAase-Sensitive Antigens by Complement Fixation with Preparation #1C Known to Contain DNAase-Sensitive Antigens.

#1C preparation, 4°C			
Antigen,* μg DNA	Antiserum S-12 1:1000	1:500	Buffer
	%		
.5	72†	58	102
1.0	43	13	102
2.0	21	0	99
Buffer	100	100	100

* U.V. absorption at 260 mμ and based on purified calf thymus DNA as standard.
† % cells lysed.

#1C preparation, DNAase, 37°C, 24 hr			
Antigen, μg DNA	Antiserum S-12 1:1000	1:500	Buffer
	%		
.5	93	96	100
1.0	89	90	102
2.0	85	24	100
Buffer	100	100	100

Summary. Complement-fixation has proved to be a rapid, sensitive method of testing DNA preparations obtained from *Brucella abortus* for DNAase-sensitive antigens, and similarly, for analyzing antisera for possible antibodies against DNAase-sensitive antigens. Considerable variation in such antigens has been found among various DNA-rich preparations isolated from the same *Brucella abortus* strain and among antisera prepared against such preparations. The percentage of rabbits that yielded antibodies against DNAase-sensitive antigens following immunization with a preparation selected for DNAase-sensitive antigens was greater than that obtained in prior studies with unselected immunizing preparations.

The efficient technical assistance of Miss Christine Pootjes, Mrs. Janet Collins, and Mrs. Janet Feder is gratefully acknowledged.

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A Modified Total Pancreatic Fistula.* (26819)

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The difficulty of maintaining dogs with a total pancreatic fistula in good electrolytic and nutritional balance is well-known(1,2). During our experiments a total fistula was required for various 24-hour collection periods and for spot testing. However, at other times there was no contraindication to returning the pancreatic juice to the animal and thus circumventing these difficulties. After the following preparation is functioning, no intravenous fluids or oral pancreas are required for maintenance:

The pancreatic ducts are located and all except the accessory duct are ligated and transected. The duodenum is transected 2-3 cm on either side of the accessory duct. A steel cannula is inserted into the duodenum containing the duct and this pancreatico-duodenal pouch is then closed. The transected duodenum is now anastomosed in continuity. A second cannula is placed in the distal duodenum, wrapped with omentum and brought through the body wall to the outside about 6 cm from the cannula of the pancreatico-duodenal pouch. A stiff-walled rubber tube is then used several days later to connect the 2 cannulae (Fig. 1).

Obstruction of the duodenal cannula by food can be controlled by doing a gastro-jejunostomy, inserting the cannula into the ileum, or constructing a one-way rubber flutter valve from a Penrose drain. The external portion of the fistula is protected by a cloth bag to prevent the dog chewing the tube.

If the pancreatico-duodenal fistula and gastro-jejunostomy are to be done, operative mortality can be reduced by doing the operation in 2 stages.

During collection periods a balloon or football-bladder on a threaded connecting tube is screwed into the cannula of the pancreatico-duodenal pouch. The duodenal cannula is blocked by a threaded plug.

Dogs can be maintained without difficulty for several months (or longer) on routine kennel diet and care. Occasional inspections must be made to remove concretions of precipitated salts from the lumen of the steel cannula. The

FIG. 1. A diagrammatic view of the preparation. During collection periods a balloon is attached to the pancreatic cannula and the duodenal cannula is capped.

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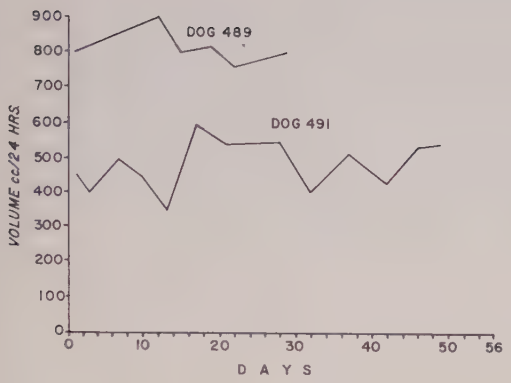

FIG. 2. Twenty-four-hr volumes of pancreatic juice from 2 dogs.

24-hour volumes of pancreatic juice of different dogs varied from 300-700 cc. However, the individual rate did not vary appreciably as the graph shows (Fig. 2).

At autopsy, there was no gross or histological evidence of duct dilatation or of pancreatitis.

Summary. This report describes a surgical procedure for preparing a pancreatico-duodenal fistula and shows the daily volume of secretion for 2 dogs.

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Antithyroid Effects of an Iodinated Hydroquinone Derivative.* (26820)

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A number of iodinated diphenyl ether derivatives have been evaluated with respect to their thyroid hormone-like or antithyroid properties(1). No simple iodinated hydroquinones or their derivatives have been evaluated however with respect to these characteristics. Since 2,6-diiodohydroquinone (DIH) has been suggested to be a potential degradation product of thyroxine(2) it was considered of interest to determine whether this simple hydroquinone or its derivatives possess hormonal or antihormonal characteristics. Preliminary studies of the effect of DIH on O_2 consumption in mice revealed that its toxicity interferred with an evaluation of its hormonal or antihormonal qualities. The diacetyl derivative of DIH (DDIH) however proved relatively non-toxic and exhibited antithyroid effects. This paper reports the extent and nature of these characteristics of DDIH.

Methods. The antithyroid characteristics of DDIH were measured through a study of its influence on the elevated O_2 consumption of mice injected with L-thyroxine or L-triiodothyronine. Methods and equipment used were essentially those of Maclagan *et al.*(3) except that exogenous L-thyroxine and L-triiodothyronine were injected at levels of 2 mg/kg and 1.68 mg/kg respectively. DDIH dissolved in cotton seed oil was injected in 2 equal portions, the first injected intraperitoneally approximately 5 hours prior to the injection of the thyroid hormone, and the second 24 hours later. Measurement of O_2 uptake was made 48 hours after first injection of DDIH.

Results. The influence of DDIH on O_2 consumption of mice is shown in Fig. 1. DDIH at a dose level of 400 mg/kg has no significant influence on O_2 consumption of untreated mice. Mice injected with 2 mg/kg of L-thyroxine, however, have significantly inhibited O_2 uptakes in presence of DDIH ($P = < 0.001$).

The influence of various concentrations of DDIH on O_2 uptake of mice is shown in Fig. 2. DDIH appears to induce a measurable effect at a total dose of 300 mg/kg. The maximum inhibitory effect appears to be at 400 mg/kg. Beyond this level the inhibitory action appears to have reached a plateau.

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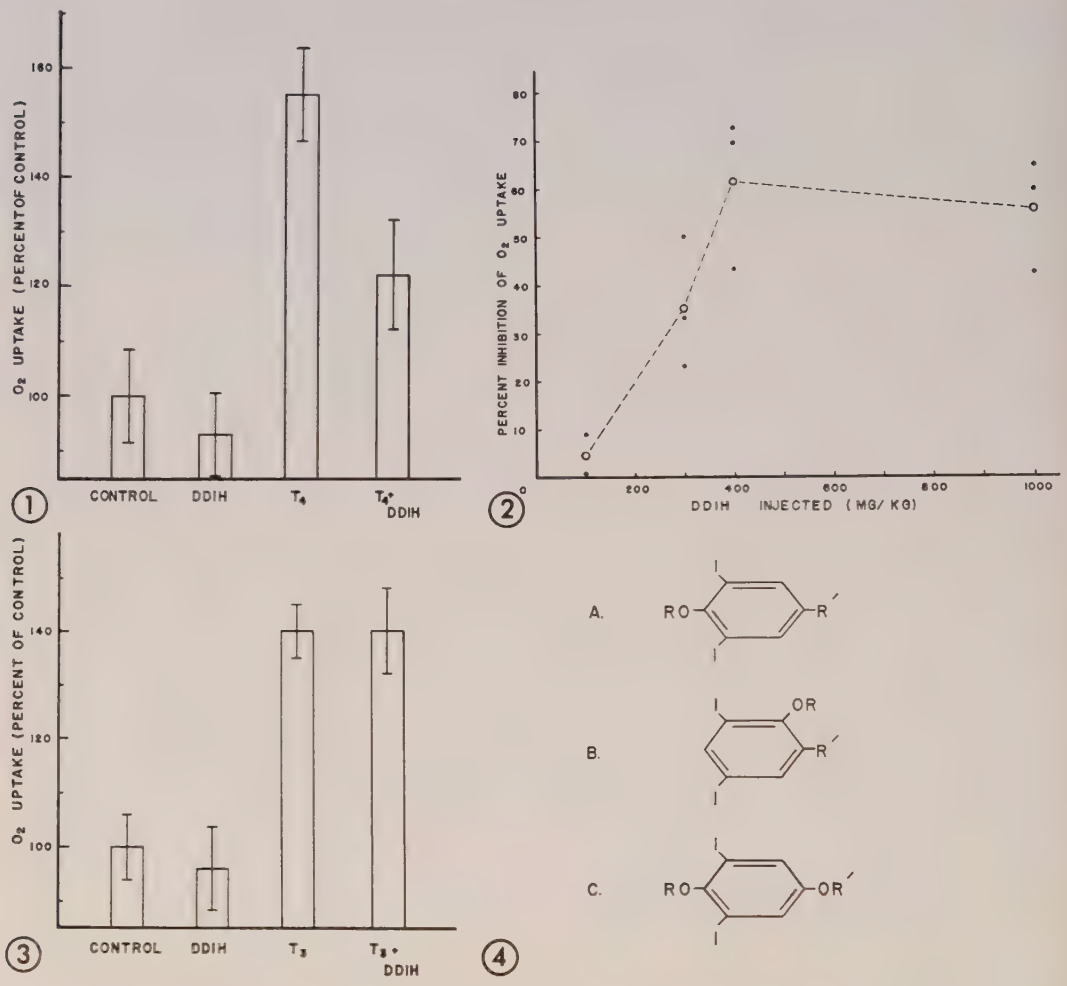

FIG. 1. Influence of a total dose of 400 mg/kg of the diacetyl derivative of 2,6-diiodohydroquinone (DDIH) on O_2 consumption of mice in presence or absence of 2.0 mg of exogenous L-thyroxine (T_4). Vertical line at top of each bar is 2 stand. deviations in length.

FIG. 2. Effect of increasing concentrations of inj. DDIH on inhibition of elevated O_2 consumption of mice receiving 2 mg of exogenous L-thyroxine. Each solid circle represents an individual reading with a separate group of 8 mice. Open circles represent avg of a given series of individual readings.

FIG. 3. Influence of a total dose of 1000 mg/kg of DDIH on O_2 consumption of mice in presence and absence of 1.68 mg of L-triiodothyronine. Vertical line at top of each bar is 2 stand. deviations in length.

FIG. IV. Generalized structures of compounds with antithyroid activities. R and R′ may be hydrogen or aliphatic groups.

The influence of DDIH on mice O_2 uptake elevated by injections of L-triiodothyronine instead of L-thyroxine is shown in Fig. 3. It is apparent that DDIH has no inhibitory action in mice injected with L-triiodothyronine.

Discussion. The observation that DDIH inhibits the elevation of O_2 uptake in mice injected with L-thyroxine but not in those injected with L-triiodothyronine suggests that DDIH functions as an antithyroid compound in the same manner as n-butyl-3,5-diiodo-4 hydroxybenzoate (BHDB). Wilkinson *et al.* (4) have theorized that BHDB acts as an antithyroid substance by virtue of its ability to prevent deiodination of thyroxine to triiodothyronine, the active form of the thyroid hormone(5).

These data do not establish whether DDIH is inhibitory *per se* or whether it must first be hydrolysed to the free hydroquinone to induce

an inhibitory response. Preliminary studies with I^{131} labeled DDIH indicate however that the major portion of injected DDIH is excreted via the urine in an unchanged form. If the compound is inhibitory *per se* this would indicate that an inhibitor of the deiodinase responsible for thyroxine degradation need not possess a free hydroxyl group. This possibility has already been suggested by earlier authors(1).

Sheahan *et al.*(6) have generalized as to the structures of compounds which exhibit BHDB characteristics. They suggest that one of structures A or B of Fig. 4 is required for BHDB-like action. Our data would indicate that structure C must now be added to this group of antithyroid substances.

Summary. The diacetyl derivative of 2,6-diiodohydroquinone has been shown to behave as an antithyroid substance. The available evidence would suggest that the compound inhibits the deiodinase responsible for conversion of thyroxine to triiodothyronine.

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Effect of Pregnenolone, Progesterone, and Derived Metabolites on Mammary Gland Growth in Rats.* (26821)

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A number of steroid compounds have been shown to have varying mammary gland growth promoting (mammogenic) capacities when injected into ovariectomized mice(1). Differences in extent of lobule-alveolar growth were evaluated by visual examination of whole mounts of glands, and criterion of response was based on percentage of mice showing a minimum amount of lobule-alveolar growth after 10 daily subcutaneous injections of material to be assayed. More recently, improvements have been made in the method of measurement of mammary gland growth of mouse (2) and rat(3), using desoxyribosenucleic acid (DNA) as an index of normal and experimental mammary gland growth. With these methods and employing a 19-day injection period (normal gestation) it is now possible objectively to quantitate amount of mammary gland growth resulting from injection of a given amount of steroid.

Recent studies(4) have established a steroidogenetic pathway involving the following transformations: pregnenolone → progesterone → 17α-hydroxyprogesterone → androstenedione → testosterone. Progesterone in synergism with estradiol has been shown to stimulate mammary gland growth in rats equal to that stimulated during pregnancy as measured by total DNA(3). It seemed of interest to determine whether pregnenolone would be converted to progesterone efficiently in ovariectomized rat as measured by mammary gland growth and further to determine whether steroids involved in the biosynthetic transformation of progesterone to androgens showed mammary gland stimulating properties which might result from these further biogenetic changes.

Materials and methods. Sprague-Dawley-Rolfsmeyer rats with an initial weight of 220-250 g were ovariectomized. Animals killed 35

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† U.S.P.H.S. postdoctoral Fellows of Nat. Inst. Health.

TABLE I. Effect of Pregnenolone, Progesterone, and Derived Metabolites on Rat Mammary Gland DNA.

Treatment	No. of animals	Body wt (g) mean	DFFT* (mg) mean	DNA (μg/mg DFFT)	Total DNA (mg/100 g BW)
Castrate controls	8	282	682	11.6	2.807 ± .22
EB, 1 μg	12	304	823	13.0	3.523 ± .18
EB, 1 μg + P, 3 mg	10	318	1499	15.4	7.009 ± .34
Idem	17	278	618	30.2	6.66 ± .46†
"	19	269	589	35.7	7.72 ± .25‡
EB, 1 μg + pregnenolone, 3 mg	9	267	824	14.7	4.535 ± .36
EB, 1 μg + 17α-OH-progesterone, 3 mg	10	316	1089	12.9	4.448 ± .17
EB, 1 μg + androstenedione, 3 mg	10	289	998	13.8	4.772 ± .42
EB, 1 μg + testosterone propionate, 3 mg	9	282	748	17.6	4.673 ± .27

* DFFT = dry, fat-free tissue. † Data from(5). ‡ Data from(3).

days post-castration served as controls. Remaining animals were divided into groups receiving 1 μg estradiol benzoate (EB), 1 μg EB plus 3 mg progesterone (P), 1 μg EB plus 3 mg pregnenolone, 1 μg EB plus 3 mg 17α-hydroxyprogesterone, 1 μg EB plus 3 mg Δ^4-androstene-3,17-dione (androstenedione), and 1 μg EB plus 3 mg testosterone propionate daily for 19 days commencing 14 days after castration. Steroids were dissolved in propylene glycol such that 0.1 ml solution was injected daily in each rat. Groups were killed one day following last injection, skinned rapidly and 6 abdomino-inguinal glands removed and frozen. DNA was determined as described previously(3). Visual observations were made for presence of lobule-alveolar development but no attempt was made to subjectively quantitate extent of growth by this method.

Results. Total DNA produced by injection of 1 μg EB daily for 19 days differed significantly from DNA observed in castrate controls ($P < .001$) (Table I). P injected in synergism with EB, produced total DNA of approximately same order as observed in previous studies, and differed significantly from DNA observed in rats receiving EB alone ($P < .001$). All groups receiving pregnenolone or metabolites of progesterone plus EB had total DNA which was significantly higher than DNA in rats receiving EB alone ($P < .05$) but lower than in rats receiving EB plus P ($P < .01$). Groups receiving EB plus pregnenolone, 17α-hydroxyprogesterone, androstenedione and testosterone propionate produced 29.0%, 26.5%, 35.8% and 33.0% of growth produced by EB plus P over that produced by injection of EB alone. Variable but definite lobule-alveolar development was observed in groups receiving EB plus progesterone, pregnenolone, 17α-hydroxyprogesterone, androstenedione, and testosterone.

Discussion. Previous study(1) has shown that a number of steroids are effective to variable degrees in production of lobule-alveolar mammary gland growth in mice. The present study confirms these findings in rats, and indicates that, of compounds tested, progesterone is most effective. One objective of this study was to elucidate configurations among naturally occurring neutral steroids necessary for stimulation of maximum mammary gland lobule-alveolar growth. Since progesterone is most active, it is evident that most potent biological activity is related to following steroid configurations: (a) C_{21} nucleus, (b) Δ^4-3-ketone, and (c) C-20 ketone. From other compounds tested, it would appear that replacement of any of these groupings with following configurations will decrease mammogenic activity: (a) Δ^5-3β hydroxyl grouping, (b) 17α-hydroxyl, (c) 17-ketone group and (d) C_{19}, C-17 propionate.

From steroidogenetic point of view, it would appear that pregnenolone could promote mammogenesis by one of two mechanisms. Either its action is attributable to partial conversion to progesterone, or it is active *per se*. In either case, it would appear that the Δ^5-3β

hydroxy grouping of pregnenolone is detrimental to the molecule's mammogenic action, for replacement of this group by the Δ^4-3 ketone group (as in progesterone) greatly increases its mammogenic potential.

There is no evidence indicating conversion of 17α-hydroxyprogesterone, androstenedione, or testosterone propionate to progesterone. Results indicated that these compounds promoted considerably reduced, although definite mammary gland lobule-alveolar development compared to EB and P. It would seem entirely possible that these metabolites of P produced in normal biogenetic pathway, may be responsible in part for mammary gland lobule-alveolar growth observed in rats injected with P.

Summary. Compounds in a steroidogenetic pathway (pregnenolone → progesterone (P) → 17α-hydroxyprogesterone → androstenedione → testosterone propionate) were tested to determine their mammogenic effectiveness (as determined by desoxyribosenucleic (DNA) measurement) in ovariectomized rat when injected with estradiol benzoate (EB). Results indicated that EB plus P produced greatest total mammary gland DNA. EB plus pregnenolone, 17α-hydroxyprogesterone, androstenedione or testosterone propionate produced 29.0%, 26.5%, 35.8%, and 33.0% of DNA produced by EB plus P over that produced by injection of EB alone.

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17-Ethynyl-4-estrene-3,17-diol Diacetate: A Unique Steroidal "Progestin".* (26822)

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In recent years the organic chemist has provided the biologist and clinician with a wide variety of synthetic steroidal substances. These have taken the form of potent estrogens, progestins and adrenal corticoids. Separation of hormonal effects has become a reality: the masculinizing properties of androgens have been decreased without reducing their anabolic effects(1,2) and separation of lipid-shifting from "estrogenic" properties of the estrogens has been achieved(3). In addition, the progesterone-like activity of the nortestosterone agents has been increased while their androgenic activities have been largely eliminated(4). This report describes a new steroid, 17α-ethynyl-4-estrene-3,17-diol diacetate, which is progesterone-like yet antiprogestational and also estrogenic as well as estrogen-antagonistic.

Methods. Progestational and progesterone-antagonistic activities of 17α-ethynyl-4-estrene-3,17-diol diacetate (EED) were studied using immature female rabbits primed with estradiol-17β for 6 days (Clauberg Assay). On the day following last injection the animals received the test compound alone (for progesterone-like activity) or in combination with progesterone (progesterone-antagonistic activity). The daily dose, in each instance, was contained in 0.1 ml corn oil and was administered subcutaneously or buccally for 5 days.

Animals were sacrificed on the day following last injection and a segment of each uterus was removed, fixed and prepared for histological examination. Degree of glandular arborization was determined and graded

* Synthesized by Drs. Frank B. Colton and Paul Klimstra, Division of Chemical Research, G. D. Searle & Co.

after the method of McPhail(5). Estimates of progestational potency were obtained from dose-response curves of the compound and standard, subcutaneously or buccally administered progesterone. Anti-progestational activity of the compound was dependent upon depression of the uterine arborization produced by a daily dose of 0.1 mg progesterone. In addition, progesterone-like activity was assessed using the technique of McGinty(6) in which the compound was administered to ligated segments of uteri in estrogen primed mature-spayed rabbits. Arborization of the glandular epithelium again served as the index of activity.

Estrogen and estrogen-antagonistic properties were obtained from studies involving *uterine growth* in intact immature female mice treated with EED alone (for estrogenic activity) or in combination with 0.3 μg estrone (for estrone-antagonistic activity)(7). The total dose was given in 3 equal subcutaneous injections over 3 successive days. The daily dose of the compound or compound plus estrone was given in a single injection of 0.1 ml corn oil. At autopsy, 24 hours after final injection, uteri were removed, cleaned, scored and blotted to remove fluids and weighed on a torsion balance. A second method for determination of estrogenic activity was the rat *vaginal smear assay*. The rats were spayed at least 30 days before being placed in the test colony. Colony rats responding positively to estrone priming on 2 consecutive weeks were chosen and placed on treatment during the third week(8). The test substance was given in 2 injections 24 hours apart and vaginal smears were taken 56 and 72 hours after the first treatment. The smears were read according to the method of Biggers and Claringbold (9) where the absence of leukocytes was considered as a positive response.

Results and discussion. 17α-Ethynyl-4-estrene-3,17-diol diacetate (EED) proved to be a potent progestin as well as a progesterone-antagonist as judged from its effects on the uterine epithelium in rabbits (Fig. 1 & 2). Comparison of the dose-response curves obtained with EED and progesterone, when both were administered buccally, revealed EED to be about 40 times more effective than progesterone (Fig. 1). Following subcutaneous administration the potency of EED was estimated to be 1 to 2 times that of progesterone (Fig. 2). Maximal arborization following subcutaneous administration was obtained at a dose of 0.1 mg per day. However, as the dose was increased above this level the response of the glandular epithelium in the uterus was reversed and was absent at the 2 and 4 mg dose levels. No such reversal was obtained with progesterone at high dose levels. Thus, EED differs from progesterone in this regard. When EED was administered buccally, no depression of the epithelial response was observed. This suggests differences in absorption and/or metabolism of EED by the 2 routes.

When administered in combination with 0.1 mg of progesterone per day, 1 and 2 mg of EED antagonized the progesterone-induced arborization of the uterine epithelium (Fig. 2). Doses of EED below 0.5 mg failed to antagonize the progesterone response but it will be noted that at these levels the compound itself was acting as a progestin. Thus, EED not only antagonizes its own progestational effects, as shown earlier, but also inhibits the response to added progesterone.

Administered locally (McGinty Assay), EED was not effective in stimulating proliferation of the glandular epithelium in rabbits (Table I). Since the hormonal effects of EED have been shown to change at different dose levels the lack of activity in this single study may be due to the dose at which it was tested. Differences in absorption at the site of action can not be discounted.

Estrogenic activity of EED was demonstrated in both bioassays to which it was subjected. It produced vaginal cornification in spayed rats; ED_{50} values for EED and estrone were 54.0 and 2.4 μg per rat respectively. Thus, the relative potency of EED at this response level was estimated to be 4% (Fig. 3). EED was found to have uterine growth-stimulating properties in immature mice (Fig. 4). However, EED differed from estrone in this study since it had a shallow (impeded) dose-response curve and did not produce maximal uterine growth. In contrast to this uterine stimulating property, EED antagonized the effects of 0.3 μg of estrone on uterine growth (Fig. 3). Maximal inhibition of the response

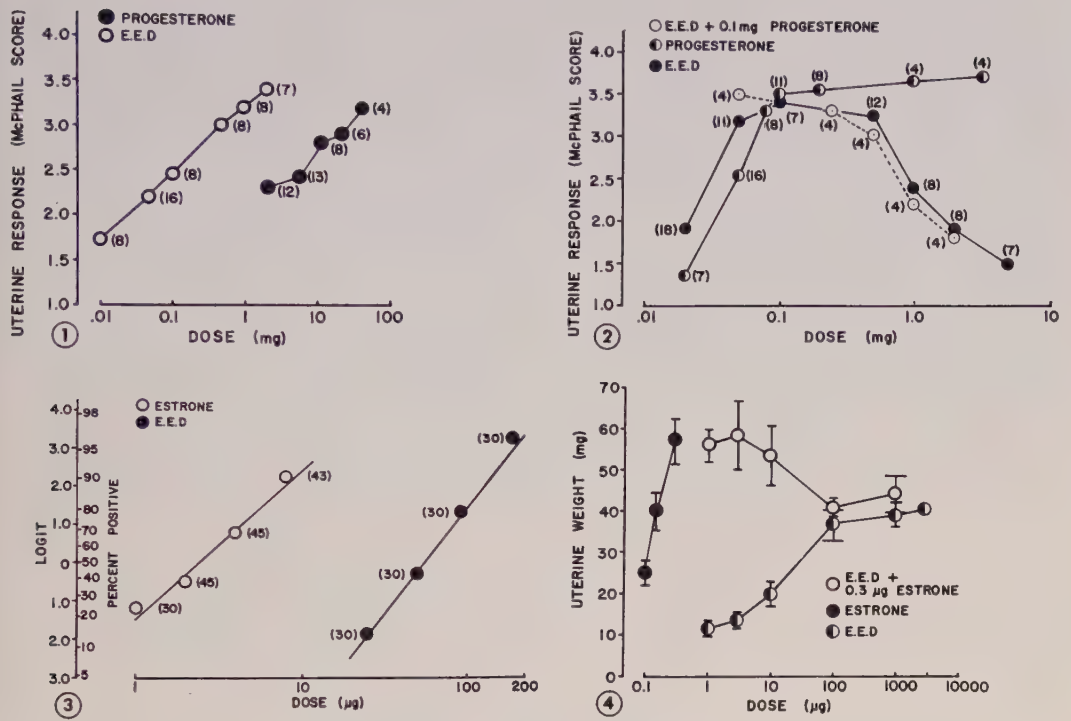

FIG. 1. Response to buccally administered EED and progesterone in uterine epithelium of rabbits. Numbers in parentheses refer to number of animals tested.

FIG. 2. Progesterone-like and progesterone-antagonistic effects of subcut. administered EED as measured by proliferative response in uterine epithelium in immature rabbits. Numbers in parentheses refer to number of animals tested.

FIG. 3. Comparison of EED and estrone on vaginal cornification in spayed rats. Dose-response lines and ED_{50} values were obtained by the minimum logit X^2 method(10). The observed responses were transformed from per cent to logit values and plotted as a function of dose. Parenthetical numbers indicate number of animals in each observation.

FIG. 4. Estrogen-like and estrogen-antagonistic effects of subcut. administered EED as measured by uterine weights in immature mice. Each point represents mean uterine weight for 8-10 mice; vertical bars represent ± 2 stand. errors of mean.

to estrone (approximately 35%) was obtained with 100 μg EED. However, doses above 100 μg failed to antagonize estrone-induced uterine growth since the uterine weights produced with these doses approach the asymptote obtained with the compound alone. It is of interest to note that in these studies both uterine growth (estrogenic effect) and estrone-antagonism (progestational effect) are obtained over the same dosage range. However, in the rabbit studies progestational and estrogenic effects were patently separated. The progesterone-antagonistic property of EED in rabbits can best be explained on the basis of its estrogenic component at the 1 to 4 mg dose levels since it was estimated to have about 4% of the potency of estrone. At lower doses the progestational component is dominant.

TABLE I. Endometrial Response to 17α-ethynyl-4-estrene-3,17-diol diacetate (EED) Administered to Ligated Segments of Rabbit Uteri.

Compound	Dose (μg)	N	McPhail score*
Oil	—	5	1.1
Progesterone	.5	7	2.3
EED	100	4	1.2
	10	4	1.6

* In our studies only values of 2 or over on the activity scale are considered positive (active); 4 represents maximal activity.

Summary. 17α-Ethynyl-4-estrene-3,17-diol diacetate is an agent that exerts unique hormonal effects. By the subcutaneous route EED is 1 to 2 times more potent than progesterone in producing proliferation of the uterine epithelium in rabbits, yet it is also capable of inhibiting this progestational response.

Buccally, EED is approximately 40 times more effective than progesterone, administered by the same route. EED is estrogenic as measured in both the mouse uterine growth assay and the rat vaginal smear assay yet when administered in conjunction with estrone EED acts as an estrogen-antagonist. These opposing activities can best be explained by the differences in hormonal dominance at low and high dose levels.

We express thanks to Mrs. Juliette Sleeper, Mrs. Kathryn Paulsen and Miss Diane Richter for technical assistance during these studies.

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Enhancement of Resistance in Mice to Staphylococcal Infection by Preliminary Treatment with a Staphylococcal Extract.* (26823)

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Protection against staphylococcal infection has been the subject of extensive clinical and experimental investigations. Among the most provocative experiments are those in which resistance is enhanced by methods which are independent to those involved in specific acquired immunity(1,2).

While investigating the properties of a staphylococcal extract which we were using as an hemagglutinating antigen, it was found that this extract had a definite resistance enhancing effect in mice against experimental staphylococcal disease. This resistance could be demonstrated after 20 hours and held for 2 heterologous strains of staphylococci as well as for the homologous strain and for one strain of *E. coli.* The extract did not visibly disturb mice and in rabbits did not behave like an endotoxin. In this study the details of these experiments are reported.

Materials and methods. Mice—Swiss mice (CFW) weighing 15-20 g were used in all experiments, except where indicated. *Staphylococci*–"Bartlett", an epidemic, 80-81 strain, resistant to penicillin, obtained from Dr. David Rogers, then at the New York Hospital, was used for preparation of the extract and except as indicated in the text for challenging the mice. For the latter purpose the organisms from an overnight culture in trypticase soy broth were obtained by centrifugation and suspended in sterile saline. The concentration of organisms per inoculum was determined by plating out serial dilutions on blood agar plates. Two other strains were used: "H"—a non typable strain sensitive to penicillin which was obtained from Dr. Stanley Schneierson, Mt. Sinai Hospital, and "Giorgio"—a coagulase positive strain which was sent by Dr. Rene Dubos, Rockefeller Institute for Medical Research.

Staphylococcal extract. 40 ml of trypticase soy broth were inoculated with a few drops of a 4 to 6 hour culture of the "Bartlett" strain of staphylococcus. After 18 hours of incubation at 37°C the culture was centrifuged and

* Supported in part by research grant E-3366 from Nat. Inst. of Allergy and Infect. Dis.

the supernate removed. It was acidified by addition of 0.5 ml of concentrated glacial acetic acid, then boiled over a hot plate until the volume was reduced to approximately 2 ml. This concentrate was passed through a Whatman #5 paper filter and added dropwise to approximately 14 ml of 95% ethyl alcohol. The precipitate was collected by centrifugation and dried under a vacuum for storage. It was reconstituted by addition of 2 ml of distilled water. If the solution was not water clear, it was centrifuged before use.

The extract was usually light brown in color. Preliminary chemical analysis, done by Dr. Anita Opper, Mt. Sinai Hospital Chemistry Dept., indicated that it contained insignificant amounts of polysaccharide and large amounts of protein and lipid. It caused no visible disturbance in mice when as much as 0.3 ml were given intravenously. Repeated subcutaneous injections caused no local reaction and did not interfere with growth of the animals. The peritoneal reaction to intra-abdominal injection was similar to that produced in response to saline injection except for a slight increase in number of polymorphonuclear cells. The extract was non-antigenic for mice since pools of serum obtained from mice 2 weeks after injection did not contain antibodies which could be detected by bacterial agglutination, hemagglutination or latex fixation. It did not behave like an endotoxin in rabbits. When 0.2 ml of twice concentrated extract were injected intradermally there was no visible reaction, nor did any appear at these sites 24 hours after 2 ml of the same material were given intravenously.

Triton X-100. This material produces a mucin-like enhancement of virulence. It is non toxic in 0.5% concentration(3).

Experimental staphylococcal disease in mice. A staphylococcal infection which produced death usually between 8 and 24 hours was produced by injecting the mice by the intra-abdominal and the intra-cerebral routes. Animals dying before 8 hours were not counted, and experiments were usually terminated at 7 days. The intra-abdominal infection was achieved with 0.5 ml of a mixture of equal parts of a saline suspension of staphylococci and 0.5% Triton X-100. The number of organisms required uniformly to kill more than 50% of the mice by this route was in the range of 10^8. The intra-cerebral infection did not require Triton. For this infection, about 10^6 organisms were given in 0.03 ml. It was usually necessary to use several dilutions of organisms in the indicated range in each experiment to attain a critical dilution. This was considered to be the highest dilution, or smallest number of organisms, which killed more than 50% of the control animals.

Results. Protection against intra-abdominal infection. In these experiments the mice were injected intra-abdominally with 0.5 ml of either saline or extract. Twenty hours later they were challenged by the same route with 0.5 ml of a mixture of equal parts of a dilution of staphylococci and 0.5% Triton X-100. Selection of the proper dilution of challenging organisms was critical. If too large a challenge was given the protective effect was obscured. However, if the effect of only the highest dilution of organisms which killed more than 50% of the control mice was studied, it soon became apparent that our extract did indeed protect. This is shown in the upper half of Table I where the results of 8 such experiments are summarized. Under the conditions of these experiments only 32% of control mice survived, as compared with 69% of the extract treated animals. The difference of 37% is highly significant.

However, since both the extract and challenge had been given by the same route, the possibility that this represented only peritoneal blockade had to be excluded. This was done by giving the challenge intra-cerebrally.

Protection against intra-cerebral infection. In these experiments, the mice were given 0.5 ml of either saline or extract intra-abdominally, and 20 hours later they were challenged by giving the organisms intra-cerebrally. In the lower half of Table I are summarized the results of 10 experiments in which critical dilutions were available. Only 24% of the control mice survived as contrasted to 56% of those given the extract. This difference of 32% is also significant, clearly indicating that the protective effect is not local but systemic.

Protective effect unrelated to size and age of mice. All of the above experiments were done with mice weighing 15-20 g. In a few experiments larger mice, mothers, weighing

TABLE I. Protective Effect of Staphylococcal Extract in 15-20 g Mice.

		Preliminary saline (.5 ml intra-abdom.)			Preliminary staph. extract (.5 ml intra-abdom.)		
Challenged:	Exp. #	No. of mice	Deaths	Survivors	No. of mice	Deaths	Survivors
Intra-abdominally (.25 ml staph. organisms + .25 ml Triton X-100)	4-32	14	12	2	13	3	10
	7-29	8	6	2	8	0	8
	8-28	13	7	6	10	4	6
	9-27	16	13	3	15	3	12
	10-26	10	6	4	10	3	7
	12-24	25	14	11	25	8	17
	14-22	15	13	2	15	6	9
	16-20	14	7	7	15	7	8
		115	78	37	111	34	77
		37/115 or 32% survivals			77/111 or 69% survivals		
		Difference of 37% $p = <.001$					
Intra-cerebrally (.03 ml of staph. organisms)	1-16	10	9	1	10	4	6
	3-13	31	24	7	31	17	14
	4-12	29	23	6	31	14	17
	5-11	31	20	11	31	13	18
	6-10	15	11	4	15	4	11
	13- 3	10	6	4	10	4	6
	14- 2	10	8	2	10	4	6
	15- 1	10	6	4	10	7	3
	16- 0	22	15	7	21	9	12
	17- 0	30	28	2	30	13	17
		198	150	48	199	89	110
		48/198 or 24% survivals			110/199 or 56% survivals		
		Difference of 32% $p = <.001$					

25-30 g, were used. In one such, after an intra-abdominal challenge, there were only 8 survivors out of 26 in the control group, while 23 of 25 extract-treated animals survived. In 2 experiments suckling mice (1 to 3 days old) were used. The amount of extract and of challenging organisms used with these small animals were different than in the other experiments. The results are summarized in Table 2. Only 8 out of 80 sucklings (10%) in the control group survived, as compared to 29 out of 83 (35%) of those which had previously received the extract.

Non-specificity of enhanced resistance. In all of the above experiments the animals were challenged with the same staphylococcus from which the extract had been prepared. In 3 experiments other organisms were used and the results indicate that this enhancement of resistance or protection is non specific. In one, the heterologous "H" strain was used to challenge the mice intra-abdominally. There were 10 mice in each group. Only 3 of the 10 control mice survived, as contrasted with 8 of 10 extract-treated animals. In a second experiment the heterologous "Giorgio" strain was used. This time the challenge was intracerebral. There were 15 animals in each group. Only 5 of the controls survived in contrast to 14 of the 15 extract-treated mice. In a third experiment an *E. coli* (0111-B4) was used without Triton to challenge the mice intra-abdominally. There were 15 animals in each group. None of the controls survived as compared with 6 of those which had been previously given the staphylococcal extract.

Discussion. The mode of action of this staphylococcal extract is unknown. Since the protective effect occurs within 24 hours, and since antibodies have not been demonstrated after 2 weeks, it is apparently not by antibody production.

North and Pawlyszyn(2) have suggested that this type of resistance is similar to interference between animal viruses. Interference is thought to operate at the level of the individual susceptible cell. The exact mechanism is unknown, but a widely held view is that it is by competition for cellular constituents which are available only in limited supply. However, the staphylococcus is not an obligate intra-cellular parasite, and so it is diffi-

TABLE II. Protective Effect of Staphylococcal Extract in Suckling Mice.

Challenged:	Exp. #	Preliminary saline (.03 ml intra-abdom.) No. of mice	Deaths	Survivors	Preliminary staph. extract (.03 ml intra-abdom.) No. of mice	Deaths	Survivors
Intra-abdominally (.03 ml staph. organisms)	11-25	44	42	2	46	35	11
	12-24	36	30	6	37	19	18
		80	72	8	83	54	29
		8/80 or 10% survivals			29/83 or 35% survivals		
		Difference of 25%			$p = <.001$		

cult to see how the enhanced resistance reported here can be called interference in the sense in which it is used in virology.

The rapid appearance of resistance might also suggest that properdin were involved, were it not for the observation(4) that gram positive cocci are insensitive to the properdin system, at least as measured by the *in vitro* bactericidal test.

A more likely mode of action for the extract is stimulation of the reticuloendothelial system. This is suggested by Bohme and Bouvier's observation(5) that the reticuloendothelial system of the mouse fails to respond in experimental staphylococcal infections, and Rowley's report(6) that extracts of *S. typhi,* which also lead to an early enhanced resistance(1), stimulate the reticuloendothelial system. However, it must be noted that our staphylococcal extract does not have the endotoxic activity of *S. typhi* extracts.

That the protective effect of the staphylococcal extract is obscured if too large a challenge is given, suggests that mice are capable only of a limited increase in this type of resistance.

Since this staphylococcal extract is relatively non toxic it offers interesting possibilities for further investigation. If it can be shown to work in other species, it may prove to be a useful prophylactic agent—not only against staphylococcal infections, but as suggested by our *E. coli* experiment, against a variety of bacterial diseases.

Summary. An extract from the supernate of an epidemic strain of staphylococcus which was not an endotoxin was injected intra-abdominally in mice. Twenty hours later the systemic resistance of these mice was significantly enhanced to lethal infections with homologous and heterologous strains of staphylococci and a toxogenic strain of *E. coli.* This effect was demonstrable in suckling, young and old mice.

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Enzymatic Activity Related to Human Serum Beta-Liprotein: Histochemical, Immuno-Electrophoretic and Quantitative Studies.* (26824)

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Histochemical technics have been successfully adapted for demonstration of enzymatic activity following starch gel electrophoresis of blood serum(1,2). In the present study these technics for 11 enzymes were applied to human serum following immuno-electrophoresis. A striking localization of activity of all 11 enzymes was found in the beta-lipoprotein precipitin line. The enzymatic activity of beta-lipoprotein fractions separated by an ultracentrifugal procedure was investigated by quantitative methods. The results suggest that beta-lipoprotein may function as a carrier of certain serum enzymes in an inactive state.

Materials and methods. Immuno-electrophoresis of human serum was carried out as described by Grabar(3). Histochemical enzyme stains were then done on the immuno-electrophoresis preparations in the same way as was previously described for starch gel electrophoresis(1,2) for the following 11 enzymes: cholinesterase, esterase, acid phosphatase, alkaline phosphatase, beta-glucuronidase, aminopeptidase, succinic, lactic, beta-hydroxybutyric and glutamic dehydrogenases and cytochrome oxidase.

Standard quantitative procedures were also carried out on whole serum and on purified beta-lipoprotein and other fractions for determination of activity of the following enzymes: transaminase(4), acid and alkaline phosphatases(5), beta-glucuronidase(6), lactic dehydrogenase(7), lipase(8), isocitric dehydrogenase(9), malic dehydrogenase(10), aminopeptidase(11), amylase(12) and cholinesterase(13).

Beta-lipoprotein (low density lipoprotein) was prepared in the Spinco Model E ultracentrifuge after adjusting the specific gravity of plasma to 1.063 with a saturated solution of equal parts of KBr and $NaNO_3$. Outdated bank blood was used as a source of plasma, but all essential findings were checked with serum or plasma freshly drawn from laboratory personnel volunteers. After centrifuging at 40,000 RPM for 12 hours the top ml of milky solution (beta-lipoprotein) was pipetted off. The bottom of the 1.063 tubes was then pooled, specific gravity adjusted to 1.21 and the pool recentrifuged at 40,000 RPM for 24 hours. The 2 ml of milky fluid at the top was carefully collected and designated alpha (high density) lipoprotein (14). Analytical ultracentrifugation was done by the method of De Lalla and Gofman (15).

Results. Histochemical methods. Fig. 1 shows enzymatic activity of the 11 enzymes demonstrated by histochemical methods in immuno-electrophoretic preparations of human serum. In each preparation whole human serum is above the trough and purified human beta-lipoprotein below with anti-human goat serum in the trough.†

The localization in all cases of enzyme activity in the beta-lipoprotein precipitin line is apparent. There is also activity in the area between the origin and the beta-lipoprotein precipitin line. Some enzymes showed activity elsewhere on the slide. Enzymatic activity in the beta-lipoprotein arc was also obtained against anti-whole serum and anti-beta-lipoprotein serum on Ouchterlony plates; examples stained for protein and 2 enzymes are illustrated in Fig. 2.

Hydrolytic enzyme controls containing no substrate or with boiled serum or beta-lipoprotein showed no enzymatic activity. In the DPN-linked enzymes for which the tetrazolium salt Nitro BT was used, minimal staining was seen in the beta-lipoprotein line

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† Hyland Laboratories, Los Angeles, Calif.

FIG. 1. Immuno-electrophoretic pattern of whole human serum (top) *vs* purified human lipoprotein (bottom). Anti-human goat serum is in trough. 1, Protein stain; 2, succinic dehydrogenase; 3, acid phosphatase; 4, alkaline phosphatase; 5, aminopeptidase; 6, beta-glucuronidase; 7, cholinesterase; 8, esterase; 9, lactic dehydrogenase; 10, glutamic dehydrogenase; 11, cytochrome oxidase; 12, beta-hydroxybutyric dehydrogenase.

in controls containing no substrate. The intensity of the stain was increased by adding substrate. The non-specific staining was entirely eliminated when boiled serum or beta-lipoprotein was used.

Enzymatic activity of beta-lipoprotein fractions. The purified beta-lipoprotein was examined for activity of the 12 enzymes listed above. Activity in all cases was below the normal serum level and the serum or plasma from which it was made, whether out-dated bank or fresh, showed levels within normal limits. After the beta-lipoprotein fraction had been exposed to sonic oscillation‡ (250 watts, 10 KC) for 40 minutes the enzymatic activity of 8 of the 12 enzymes tested was well above the normal serum level although the lipoprotein fraction represented only the top one ml from a 10 ml sample of normal serum following ultracentrifugation. Cholinesterase and alkaline phosphatase were markedly increased in the beta-lipoprotein fraction by sonic oscillation but the activity did not exceed normal serum levels. Aminopeptidase

‡ Raytheon Mfg. Co., Waltham, Mass. Sonic oscillator.

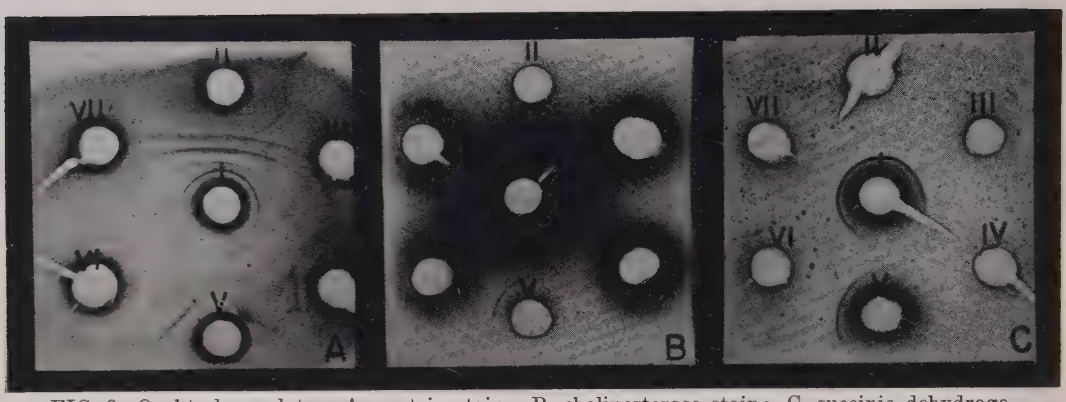

FIG. 2. Ouchterlony plates. A, protein stain; B, cholinesterase stain; C, succinic dehydrogenase stain. I, human serum; II, anti-human goat serum; III, anti-human beta-lipoprotein goat serum; IV, anti-human beta-2 macroglobulin (Waldenström) goat serum; V, human beta-lipoprotein + II; VI, II + IV; VII, III + IV.

activity was not affected by sonic treatment and no amylase activity was found either before or after treatment (Table I). Analytical ultracentrifugal analysis of the beta-lipoprotein fraction before and after sonic treatment is shown in Table II.

The effect of sonic oscillation is illustrated by the transaminase results (Fig. 3). Purified beta-lipoprotein showed activities varying from 3 to 10 units per ml in various preparations. After 40 minutes in the sonic oscillator this activity had increased to over 50 units per ml. After storing for several days the activity was again reduced, and could be restimulated to even higher levels by reexposure to sonic frequencies.

Freezing and thawing, addition of dinitrophenol, ether extraction, and bubbling oxygen through the mixture were also effective to varying degrees in liberating enzyme activity from the purified lipoprotein.

High density lipoprotein (s.g.>1.063) showed no activity either by quantitative technics or immuno-electrophoresis.

Discussion. The sequestration of enzymatic activity, as well as its liberation by physical methods, in beta-lipoprotein, is similar to observations on the enzymatic activity of mitochondria(16). Mitochondria are also known to contain lipoprotein(17). Apparently the relationship of beta-lipoprotein to mitochondria has never been investigated.

A possible interpretation of these findings is that beta-lipoprotein may form a relatively unstable complex with certain molecules whose activity is normally intracellular thereby carrying them in the circulatory system in an inactive state. The complex is probably a

TABLE I. A Comparison of Activity of Purified and Activated Beta-Lipoprotein with Whole Serum.

Enzyme	Activity			Units	Ref.
	Serum units	Purified beta-lipoprotein†	Activated beta-lipoprotein†		
Transaminase SGO-T	8 - 40/ 1 ml	5	150	Karmen	4
" SGP-T	5 - 35/ 1 ml	7	130	"	4
Acid phosphatase	0.5 - 1.5/100 ml	0	6	Bodanski	5
Beta-glucuronidase	42 - 319/ 1 ml	20	450	Fishman	6
Lactic dehydrogenase	100 - 350/ 1 ml	50	500	Sigma	7
Lipase	0 - 1/ 1 ml	0	3	Tietz, *et al.*	8
Isocitric dehydrogenase	47 - 264/ 1 ml	20	350	W-WA	9
Malic "	42 - 195/ 1 ml	35	300	Siegel, *et al.*	10
L-Aminopeptidase	75 - 230/ 1 ml	75	75	G - R	11
Cholinesterase	40 - 80/ 1 ml	±	25	Rappaport	13
Alkaline phosphatase	2 - 4.5/100 ml	0	2	Bodanski	5
Amylase	80 - 150/100 ml	0	0	mg glucose liberated	12

† Avg of 2 or more determinations.

TABLE II. Ultracentrifugal analysis (mg/100 ml) of Beta-Lipoprotein Fractions.

	Sf° 0-12	Sf° 12-20	Sf° 20-100	Sf° 100-400
A	145.4	0	4.8	5
A*	37.7	0	14.9	25
B	134.5	4.8	4.8	5
B*	32.3	0	9.9	10

A. Prepared from plasma from outdated bank blood.

B. Prepared from fresh plasma.

* After 40 min. in sonic generator.

physical rather than a chemical one, inasmuch as it can be easily dissociated by physical means. Reinactivation of the enzyme-lipoprotein mixture on standing and liberation of the enzyme activity again on reexposure to sonic frequencies would seem to support this as would the fact that certain antigen-antibody reactions are potentiated by freezing and thawing(18). The term "sequestration" is proposed for this property of beta-lipoprotein.

Summary. Activity of 17 different enzymes was found to be present in human serum beta-lipoprotein by either standard quantitative or histochemical-immunoelectrophoretic technics. By the former methods the activity could be increased by several physical procedures which apparently affected the beta-lipoprotein molecular structure. The histochemical technics were annulled by boiling and were substrate specific except for slight reactions of the DPN dependent enzymes without substrate. Staining which occurred in these cases was more intense, however, when substrate was added. These observations suggest a possible new theory of beta-lipoprotein function: namely, that it may form a complex with enzymes as well as certain other molecules whose activity is normally intracellular thereby inactivating them while in the circulatory system.

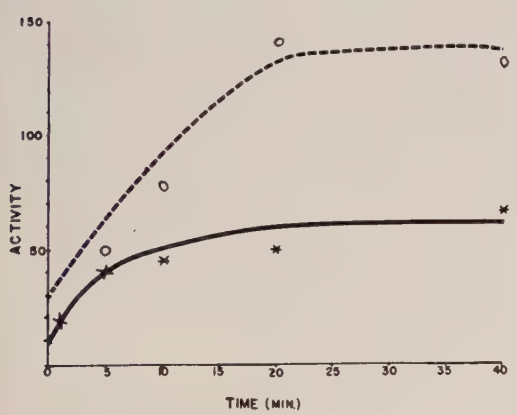

FIG. 3. Effect of sonic oscillation on transaminase (SGO-T) activity of beta-lipoprotein. Solid line, first exposure; broken line, re-exposure after 1 wk in cold room.

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Reactivation of Arthus-Type Inflammation by Reduced Glutathione. (26825)

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Recent reviews have summarized the evidence for the activation of proteolytic enzyme systems as a common mechanism for various types of allergic reactions(1,2). We found that the activity of an SH-dependent skin protease increases simultaneously with development of the cutaneous Arthus phenomenon and decreases as the inflammation subsides. The latter phenomenon is due, at least in part, to local accumulation of a protease inhibitor of polypeptide nature(3-5). Both enzyme and inhibitor are released from mesenchymal connective tissue cells and can be extracted in the euglobulin fraction of the inflamed skin(5-7). The Arthus reaction was shown to be inhibited by the inhibitor-containing euglobulin(8) or the partially purified inhibitor.

It was observed in a preliminary study that the euglobulin extracted from healing Arthus sites shows little protease activity but becomes proteolytic on addition of reduced glutathione (GSH). The present paper shows that intradermal injection into completely healed Arthus sites results in rapid reactivation of the inflammatory process.

Materials and methods. Adult male albino rabbits were sensitized with 3 subcutaneous injections of 5 ml bovine serum every other day. On the 11th day after last injection the precipitin titer of the sera was measured by the ring test(5). Animals showing precipitin titers of 2^6 were given 4 intradermal injections of 0.2 ml antigen in the flanks. The reaction became visible 5 hours later, reached its peak in 24 hours and decreased thereafter. Inflammatory reactions were graded according to the method of Cochrane and Weigle(9). The present study was done 12 to 19 days after injection when the reaction had completely disappeared.

A commercial preparation of GSH was used after verification of its reduced state by amperometric titration(10). Amounts of 0.2 or 0.3 ml of 0.1 M GSH (in physiological saline) were injected intradermally into the center of 2 of the healed sites, shaved 24 hours prior to injection. The 2 opposite sites received the same volume of saline. The reactions produced were compared macroscopically and histologically after fixation in 10% formol and hematoxylin-eosin staining. An injection of GSH was also made into a virgin skin site of each animal.

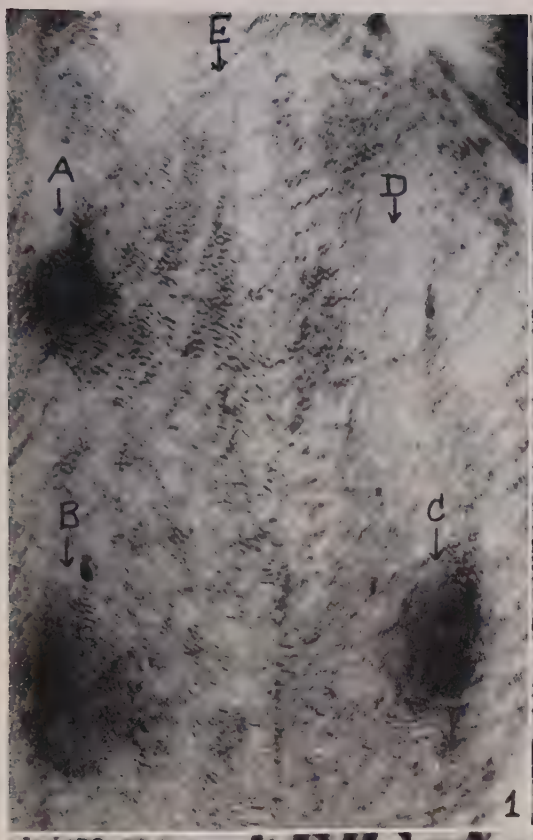
Results. Injection of GSH into healed Arthus sites was followed in all cases by the appearance of visible inflammatory reactions within 2 hours, which reached a peak in 9 to 11 hours and lasted 72 to 96 hours. These reactions developed and regressed more rapidly than the original Arthus phenomenon. At the height of the reaction, moderate to severe hemorrhage was observed with mild sloughing in the center of the lesion; there was no severe desquamation or necrosis. In 3 out of 8 cases the inflammation was almost as intense as in the original Arthus reaction; in the other 5 cases the reactions were moderate (Fig. 1). With 0.2 ml GSH the inflammatory reaction followed the same sequence as above but the reaction was less marked; mean value of the great diameter of the lesion was 22 mm. In Table I is a comparison between the original Arthus reaction

FIG. 1. Reactivated inflammation in rabbit skin. A, B and C, 18-day-old healed Arthus sites inj. with 0.3 ml 0.1 M GSH (3+ grade reactions); D, healed site inj. with 0.3 ml saline; E, virgin site inj. with 0.3 ml 0.1 M GSH. Picture taken 10 hr after inj.

FIG. 2. Photomicrograph of reactivated lesion. Hematoxylin-eosin staining; ×45. Skin specimen collected 10 hr after inj. of GSH. The reaction is almost indistinguishable from original Arthus phenomenon.

FIG. 3. Marked degree of perivascular infiltration of mononuclear cells in proximity of the muscular layer. Hematoxylin-eosin, × 400.

FIG. 4. Fibrinoid exudation and leucocytic infiltration in reticular layer; small blood vessel showing lesions of panarteritis nodosa type. Hematoxylin, ×150.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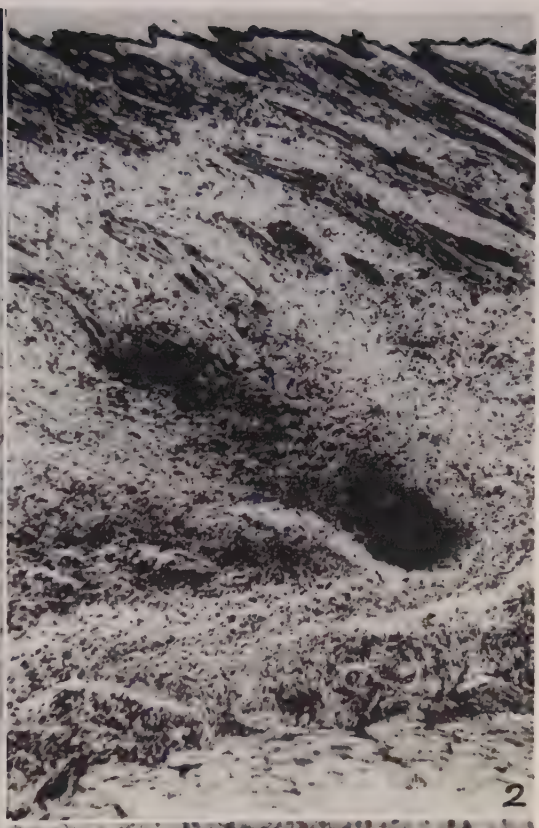
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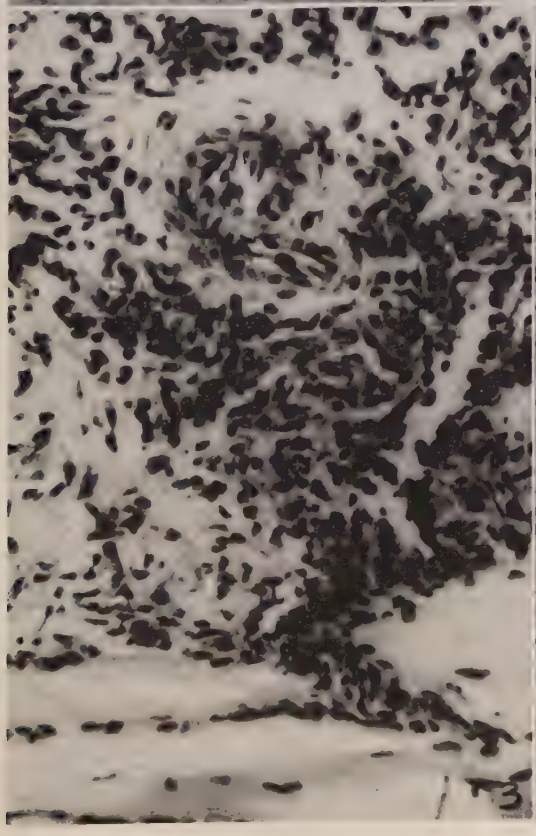
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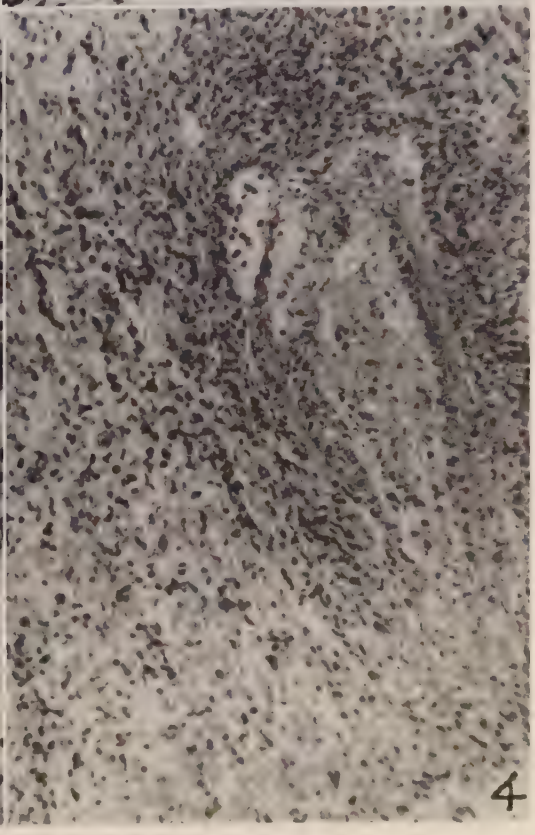
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TABLE I. Macroscopic Reactions to Injection of GSH into Healed Arthus Sites.

Animal groups No.	Inj. GSH, cc	Reactions* Original Arthus†	Healing day	GSH-inj. site‡
1, 2, 3	.3	r. 4+, 4+, 4+	12, 12, 14	4+, 4+, 3+
4, 5, 6§	"	r. 4+, 4+, 4+	15, 14, 18	2+, 2+, 3+
7, 8	"	r. 4+, 4+, 4+	19, 13	2+, 2+, 2+
9, 10, 11	.2	r. 4+, 4+, 4+	15, 18, 15	1+, 2+, 1+

* Mean grade of inflammation in each rabbit; there was little difference in reaction intensity in different sites of the same rabbit.

† Reactions at 24 hr.

‡ " " 10 hr.

§ Shown in Fig. 1.

Inj. of GSH into virgin site or of saline into healed site induced no reaction.

and the reactivated phenomenon.

No reactions were observed after injection of GSH into virgin skin sites of sensitized animals or after injection of saline into healed Arthus sites.

Histological studies on biopsy specimens taken 10 hours after injection of GSH showed varying degrees of cutaneous lesions similar to those of the original reaction. The lesions were characterized by: 1. diffuse and local infiltration by inflammatory cells; 2. fibrinoid exudation as shown by eosinophilic swelling of collagen bundles and interfibrillar deposition of an eosinophilic amorphous material; 3. vascular changes visible as eosinophilic swelling and necrosis of vessel walls; 4. hemorrhage or formation of hyaline and fibrinous thrombi in the small blood vessels. Varying degrees of these lesions were found in all sites. The most severe reactions (3 animals) were almost indistinguishable from the original Arthus phenomenon (Fig. 2). In 8 other cases the reactions were moderate and 2 among these were characterized mostly by extensive fibrinoid exudation.

The type of cells participating in the reaction is of interest. Mononuclear infiltration appeared either in a diffuse or a focal (perivascular) pattern with a preference for the papillary layer of the corium and the subcutaneous tissue close to the muscular layer (Fig. 3). Some of the cells were lymphocytes but most of them were monocytes and histiocytes. Large number of plasma cells were found in the same area, especially around small blood vessels. The dense mononuclear accumulation resembled the picture observed at a late stage of the Arthus phenomenon. Polymorphs and eosinophiles were also distributed in either a diffuse or a focal pattern but the greatest accumulation took place in the reticular layer of the corium. Fibrinoid exudation and vascular reaction also predominated in this area where varying degrees of lesions of the peri- or panarterites type were observed (Fig. 4). These lesions were similar to those observed in the early phase of the Arthus reaction.

Varying numbers of mitoses were observed in the epidermis of the reactivated area, especially in the 3 cases which showed the most intense reaction. Mitoses were also seen in the muscular layer of the walls of small and middle sized arteries.

No significant histological changes were observed in the saline injected sites or the virgin sites treated with GSH.

Discussion. Cochrane *et al.*(11) showed that reinjection of the antigen into a healed Arthus site induces a lesion similar to the original reaction. This reactivation is certainly different from the phenomenon described in this paper, and it was shown to be produced by an antigen-antibody reaction in the blood vessel walls. Our observations can probably be interpreted in terms of activation of proteolytic enzymes by GSH. It is also possible that GSH acts on the inhibitor polypeptide mentioned above since it has been shown that this substance loses its activity under the influence of reducing agents(12). GSH is a powerful endogenous hydrogen carrier and it is believed that one of its functions is to keep SH-dependent enzymes in the

reduced form. The observations presented here may have some bearing on the mechanism of the recurrence of chronic inflammatory processes in rheumatic myocarditis and rheumatoid arthritis.

Summary. Injection of GSH into recently healed sites of Arthus phenomenon produces a reactivation of the inflammatory process. The reactivated lesion has the same aspect and same histological characteristics as the original reaction.

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